STUDIES ON THE FUNCTION AND METABOLISM OF COPPER [1, 2]

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TEN FIGURES

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The adult human body contains some 100 to 150 mg of copper, of which 64 mg are found in the muscle mass, 23 mg in the bones and 18 mg in the liver. The concentration of copper is higher in the liver than in any other organ. In fetal liver it is 5 to 10 times that of adult liver (Cartwright, '50).

Copper is found in various foodstuffs, the amount in agricultural products depending on the copper content of the soil. In descending order of concentration, copper is found in nuts, dried legumes, cereals, dried fruits, poultry, fish, animal tissues and so forth (Cartwright, '50).

This element has interested physicians for many centuries. Nevertheless, even today relatively little is known about its functions. Copper was recognized as a normal constituent of blood 75 years ago (Cartwright, '50). Fifty years later, investigators at the University of Wisconsin (Elvehjem, '35) demonstrated that this element is concerned in erythropoiesis. Not long afterwards it was shown that copper is an essential dietary factor for maintaining the color of fur in several

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species of animals. It was observed (Keil and Nelson, '31) that black and piebald rats turn gray when fed a diet deficient in copper. Copper is also necessary for the folding of the keratin molecule to produce the normal structure of the wool in sheep (Marston and Lee, '48) and it is important in maintaining the myelin of the central nervous system. Lambs grazing in parts of Australia where the soil is deficient in copper have been observed to develop a slowly progressive ataxia which is quite apparent by the age of three to 6 weeks (Bennetts and Beck, '42). If this "enzootic ataxia" is treated with copper early enough, the ataxia is relieved, but if the copper deficiency is not corrected a diffuse, symmetrical, cerebral demyelination develops which is most marked in the corpus callosum, the internal capsules, the white matter of the occipital and frontal lobes and the motor pathways in the spinal cord.

Nothing is known about the manner in which copper serves these important functions. It has been shown that copper forms an essential part of the molecule of at least three plant oxidases; namely, tyrosinase, laccase and ascorbic acid oxidase. That copper is a component of cytochrome oxidase is a possibility that has not yet been eliminated. Schultze ('41) found that copper deficiency causes a great decrease of the cytochrome oxidase activity of the bone marrow of rats and concluded that a close correlation exists between the cytochrome oxidase activity of the bone marrow and its ability to form hemoglobin and erythrocytes.

Mann and Keilin ('38) described two copper proteins in human tissues, hemocuprein in the blood and hepatocuprein in the liver. Holmberg and Laurell ('51a) have suggested that 4 molecules of hemocuprein form one molecule of ceruloplasmin, a copper protein present in blood which will be discussed later. They found ceruloplasmin to be a true oxidase and classify it as a laccase. Another copper compound found in human tissues is tyrosinase (Fitzpatrick et al., '50).

Our laboratory has been interested in copper, particularly its relation to hemopoiesis, for a number of years. We pro-

pose to summarize here some of the observations which have been made to date.

THE BLOOD COPPER

Copper is normally found in the blood in both the plasma and the red corpuscles (Lahey et al., '53a; see table 1 of present paper). An insignificant amount is held in the white cells and platelets. There is a small diurnal variation in plasma copper and a slight variation in red cell copper but these are of small magnitude as compared with the diurnal variations in plasma iron. The variations from day to day and from week to week and the alterations in relation to meals or with menstruation are insignificant.

TABLE 1

Blood copper values in normal human subjects

SEX	NO. SUBJECTS	COPPER		RBC COPPER	
		Whole blood	Plasma	No. subjects	
		μg % [1]	μg % [1]		μg % [1]
Male	40	96 ± 13	105 ± 16	19	110 ± 16
Female	23	100 ± 11	116 ± 16	12	122 ± 29
Total	63	98 ± 13	109 ± 17	31	115 ± 22

[1] Figures refer to mean ± standard deviation.

In pregnancy, an increase in plasma copper occurs (Fay et al., '49). This begins early in pregnancy and continues to rise, to reach levels at term approximately twice the normal. The red cell copper content does not change.

Hypercupremia, however, occurs in many other circumstances (Lahey et al., '53b). An increase in plasma copper is observed in most acute and chronic infections. This begins soon after the onset of the infection and the level returns to normal during convalescence. In acute leukemia, a sharp fall in plasma copper from hypercupremic levels may be observed during hormone therapy, whether or not improvement takes place.

Hypercupremia has also been found in chronic leukemia, Hodgkin's disease, various types of anemia, liver disease, "collagen" disorders and other conditions (table 2). Curiously enough, iron deficiency in infants appears to be associated with much more striking increases in plasma copper than iron deficiency in adults.

In view of the claims that copper therapy is often useful in the management of hypochromic microcytic anemia in infants, it is noteworthy that in not one of 24 cases were we able to demonstrate hypocupremia in infants. In fact, there is no record in the literature of true copper deficiency occurring in man. None of the studies which have been made from time to time has demonstrated this. In a study of repatriated prisoners of war who had suffered from starvation we were unable to demonstrate hypocupremia even in those in whom hypoferremia was present (Cartwright and Wintrobe, '46). We have fed diets identical to those which were successful in producing copper deficiency in rapidly growing swine to two infants, 5 days and 8 months of age, respectively. Although this experiment was continued for 4 and 5 months, respectively, no evidence of copper deficiency developed. This may have been due to the fact that the growth of infants is less rapid, or that their copper stores are greater than in young swine; or perhaps the human requirement for copper is less than that of swine. In any event, our experience suggests that copper deficiency must be very rare in man. This does not deny, however, that copper is essential to man nor is it intended to imply that copper deficiency can never occur in the human species. There is an interesting possibility that the "curse" which led to the disappearance of human inhabitants on Kangaroo Island, which lies off the south coast of Australia, may have been copper deficiency (Bauer, '52).

Hypocupremia is very uncommon. However, there are two conditions which have been shown to be associated with hypocupremia and are also accompanied by increased urinary excretion of copper. These are lipoid nephrosis and hepatolenticular degeneration (Wilson's disease). Both conditions

TABLE 2

Blood copper in various clinical conditions

CONDITION	NO. SUBJECTS	WHOLE BLOOD COPPER	PLASMA COPPER	CELL COPPER	VOLUME OF PACKED RBC	PLASMA IRON
		μg %	*μg %*	*μg %*	*ml/100 ml*	*μg %*
Normal	63	98 ± 13	109 ± 17	115 ± 22	47	115 ± 42
Pregnancy	30	169	222	130	37	91
Infection	37	141	167	116	41	57
Acute leukemia	19	195	236	98	27	171
Chronic leukemia	21	119	148	101	39	113
Hodgkin's disease	14	142	171	109	40	78
Pernicious anemia	10	111	121	98	27	173
Aplastic anemia	8	130	152	86	28	203
Iron defic. anemia						
Adults	9	114	132	109	30	26
Infants	24	155	168	152	28	31
Hemochromatosis	14	103	134			234
Hyperthyroidism	7	108	140	70	46	124
Hypothyroidism	7	105	126	103	37	83
Wilson's disease	3	79	55	110	42	64
Nephrosis	3	70	80	119	44	62

clearly deserve more intensive study from this point of view. It has been demonstrated that there is in Wilson's disease a marked increase in the concentration of copper in the liver as well as in the involved areas in the brain (Cumings, '48).

While plasma copper increases in many conditions, the red cell copper content tends to be constant. Certain exceptions have been observed, however. As will be shown later, red

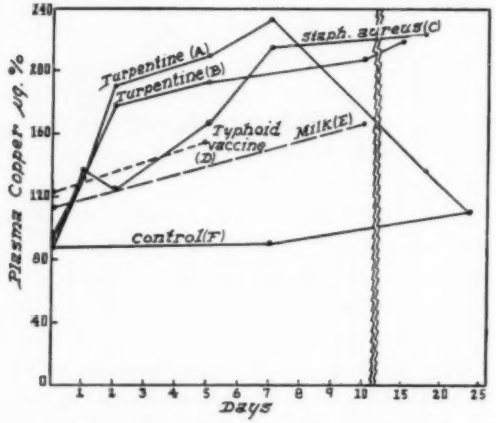

Fig. 1 The influence of turpentine, a bacterial abscess, typhoid vaccine and milk on the plasma copper level.

Group A, 0.2 ml of turpentine intramuscularly (I.M.) on day 0; group B, 0.2 ml of turpentine I.M. on days 0, 4 and 8; group C, 1.0 ml of a broth culture of *S. aureus* I.M. on day 0; group D, 0.5 ml of typhoid vaccine I.M. on days 0 and 3; group E, 0.5 ml of milk I.M. on days 0, 2 and 6; group F, no injections.

cell copper is reduced in copper deficiency. In pernicious anemia we have found the copper content of the average red cell to increase proportionately to its increased size. In hypochromic microcytic anemia in adults, the red cell copper is reduced in proportion to the microcytosis. However, in infants with hypochromic microcytic anemia, the concentration of copper in the red cells was found to be increased; that is, the size of the average red cells was more reduced than was their copper content.

It has been claimed that there is a correlation between plasma copper and the basal metabolic rate. We have not been able to confirm this (Gubler et al., '53a) but we have observed a decrease in red cell copper in several cases of hyperthyroidism (Lahey et al., '53b).

Several observations concerning factors which influence the plasma copper level in experimental animals (Gubler et al., '53a) may be described at this point. When copper is eliminated from the diet of rats, there is an early and sharp fall in the plasma copper. In figure 1 are shown variations in plasma copper in rats in which inflammation was produced

TABLE 3

The influence of dietary copper on the hypercupremia induced by inflammation

GROUP	NO. OF RATS	DIET	TURPENTINE	DAY	PLASMA Cu	LIVER Cu
					μg %	μg %
I	10	Milk	—	0	100 ± 5.4 [1]	5.0 ± 0.15
II	10	Milk	—	5	58 ± 6.3	4.1 ± 0.24
III	10	Milk	+	5	81 ± 4.1	4.2 ± 0.22
IV	10	Milk + Cu	—	5	130 ± 4.7	5.8 ± 0.10
V	10	Milk + Cu	+	5	196 ± 6.3	7.5 ± 0.52
VI	6	Stock	—	0	107 [2]	5.1 [2]
VII	6	Stock	+	5	208 [2]	4.8 [2]

[1] Figures represent mean ± standard error.

[2] Pooled samples.

by the injection of turpentine once (A) or several times (B), staphylococcus aureus (C), typhoid vaccine (D), or milk (E). In each instance hypercupremia developed.

When turpentine is injected in rats fed a copper-deficient diet, some rise in plasma copper takes place nevertheless (table 3). When copper is supplied and turpentine is injected, a very marked increase in plasma copper occurs. It is noteworthy that the copper concentration in the liver seems to have little relation to the hypercupremia which develops. One wonders whether the injection of an agent such as turpentine in some way lowers the intestinal barrier to copper, leading to its rapid absorption and passage to the liver, where more ceruloplasmin is formed.

The serum copper protein, according to Holmberg and Laurell ('47), is a blue copper-containing alpha globulin of 151,000 molecular weight and containing 8 atoms of copper. They have named this substance "ceruloplasmin" and have suggested that it contains 4 units of the hemocuprein of Mann and Keilin ('38).

When plasma copper and the plasma proteins, measured both chemically and electrophoretically, are compared, a high degree of correlation is found to exist between the serum copper and the $\alpha_2 + {}_3$-globulin in human subjects (Lahey et al., '53c). The correlation between serum copper and $\beta_1 + {}_2$-

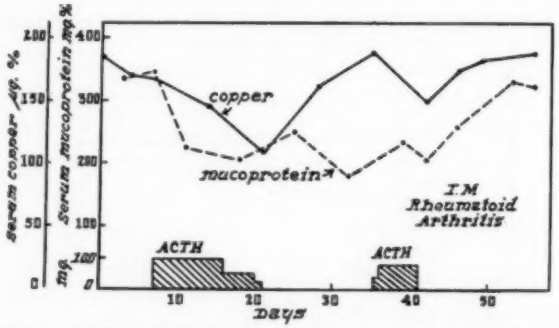

Fig. 2 The relationship between the copper concentration and the mucoprotein concentration in human sera.

globulin is less good, while the correlation with albumin is poor. We could demonstrate no significant correlation with total protein, α_1-globulin or γ-globulin. Since plasma copper tends to be increased in conditions in which the erythrocyte sedimentation is rapid, it was of interest to compare variations in plasma copper with an acute phase reactant such as mucoprotein. In figure 2 variations in serum copper and in mucoprotein in a case of rheumatoid arthritis treated with ACTH are shown. It is evident that there was no definite correlation.

In rat blood the correlation is high between serum copper and α_1-globulin and between serum copper and $\beta_1 + {}_2$-globulin.

From these observations one might assume that serum copper is bound to an α-globulin. This is consistent with the observations of Holmberg and Laurell already mentioned.

The functions of ceruloplasmin are unknown. Holmberg and Laurell showed that it serves as an oxidase ('51b). We have attempted to determine whether ceruloplasmin transports newly absorbed copper from the gastrointestinal tract to the liver (Gubler et al., '53b). The results of these studies may be described briefly.

TABLE 4

Total and direct-reacting plasma copper

	HUMAN		RAT		DOG	PIG
	Normal	Pregnant	Normal	Turpentine-injected	Normal	Normal
Number	7	8	10 [1]	10 [1]	7	2
Plasma Cu, μg %	110	228	107	208	77	172
Direct-reacting Cu, μg %	4	18	1	3	9	72
% of total Cu reacting directly	4 (0–17)	8 (0–17)	1	1	12 (0–27)	42 (40–43)

[1] Pooled specimens.

When copper is added directly to serum *in vitro,* it reacts with diethyldithiocarbamate. This we have termed "direct reacting copper." The copper in ceruloplasmin does not react in this way. It must first be liberated from the protein by acid. It is, therefore, referred to as "indirect reacting copper."

Data giving the quantity of direct reacting copper in the plasma of several species of animals are shown in table 4. In human plasma only 4% of the plasma copper is direct reacting. About twice this amount was found in the plasma of pregnant females with hypercupremia. In normal rats and in animals in which inflammation had been produced by the

injection of turpentine, only 1% of the copper was direct reacting. In dogs, 12% was direct reacting, while in pigs as much as 42% was of this type.

It is noteworthy that the ratio of direct to indirect reacting copper remains the same even in the plasma of a copper-depleted animal. The plasma of the animal depleted of copper is not capable of binding more than the usual quantity of

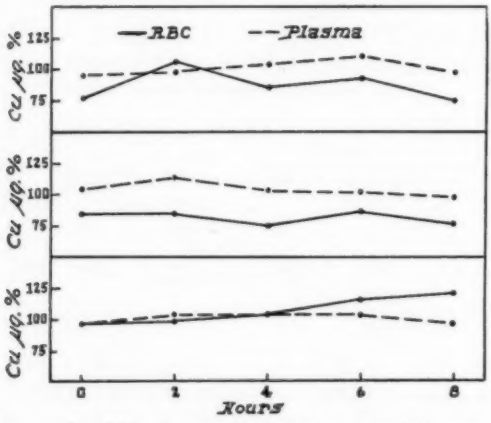

Fig. 3 Plasma and red blood cell copper in three normal human subjects following the oral administration of 50 mg of copper in the form of "Cupralene."

copper. This is in contrast with the avidity for iron manifested by the plasma iron-binding protein in the presence of iron deficiency.

In figure 3 the blood levels in three human subjects given 250 mg of cupralene orally (allycuprothiocarbamide, containing 19.93% copper) are shown. No significant change in plasma or red cell copper took place. When much larger amounts of copper are given by mouth, an increase can be demonstrated. One hundred and sixty-five milligrams of copper per kilogram body weight were given as copper sulfate to a dog. There was a sharp rise in plasma copper, due entirely to the appearance of the direct reacting form. Subse-

quently a very striking increase in copper content of the red cells took place. This dose of copper was toxic.

When copper was given intravenously in man (fig. 4), the plasma copper rose sharply as the result of the appearance of direct reacting copper, but the copper disappeared completely in 4 hours. This was due to the disappearance of the direct reacting form. It is of interest that a significant increase in red cell copper occurred as well.

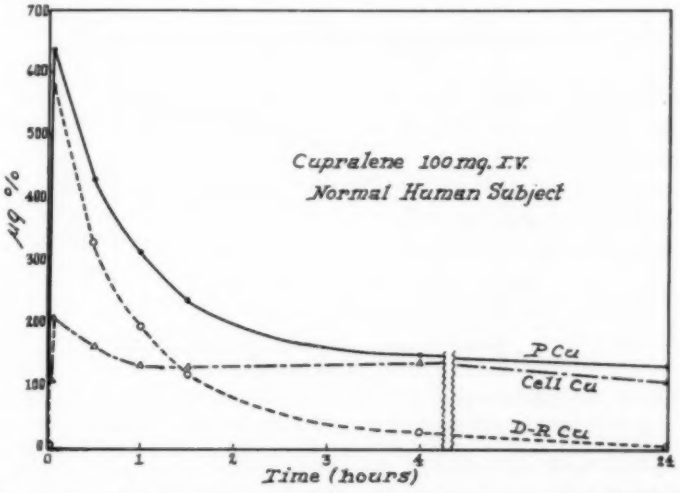

Fig. 4 Total plasma copper (P.Cu), direct-reacting plasma copper (D-R Cu) and red cell copper (Cell Cu) in a normal human subject at various intervals of time after the intravenous administration of 20 mg of copper in the form of "Cupralene."

Indirect reacting copper does not transfer from plasma to red cells, no matter how high the plasma copper. On the other hand, the direct reacting copper transfers readily. For example, in an experiment in which copper sulfate was injected intravenously in a dog and the blood was withdrawn and incubated, the copper was found to shift from the plasma to the red cells (fig. 5). Such red cells hold their copper avidly. Red cells containing increased copper, when incu-

bated with normal plasma, reveal no shift. The movement thus seems to be only in one direction.

These observations suggest that the copper which is present in the plasma of human, rat, dog and pig blood is bound to protein in two different ways. The so-called indirect reacting copper makes up the greater proportion of the copper in the plasma and is firmly bound. The quantity of such firmly bound copper is relatively stable under normal conditions. It is increased in a variety of abnormal conditions and is

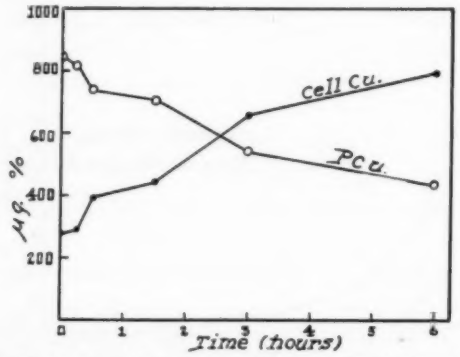

Fig. 5 Transfer of copper from plasma to red cells during incubation of blood. A normal dog was given 0.5 mg of copper per kilogram of body weight intravenously in the form of copper sulfate. Two minutes following the injection, a sample of blood was withdrawn from the other jugular vein and incubated for 6 hours at 37°C.

rarely decreased below normal. On the other hand, except in swine, the amount of direct reacting copper is very small. How the direct reacting copper is bound in plasma is not known, but it seems likely that the albumin, the β_1, metal-binding globulin, and other proteins may carry it. It would seem plausible that the direct reacting form of copper is copper in transit on the way to the liver, the principal storage site.

The contrast with transferrin, the iron-binding protein of the plasma, is interesting. This protein is normally only one-third saturated and is ready to bind more iron. The iron

in the plasma appears to be entirely in transport, serving no function at that stage. The copper in plasma, on the other hand, is probably mainly functional and perhaps serves as an active enzyme. More mysterious is the role of the copper in red cells. Probably none of this copper represents copper in transit.

There is no evidence that plasma copper is related in any way to erythropoiesis. There is no correlation between hypercupremia and hyper- or hypoferremia.

HEMATOLOGICAL MANIFESTATIONS OF COPPER DEFICIENCY IN SWINE

Copper deficiency was produced in some 70 pigs by feeding canned milk and water, the latter having been deprived of any copper by precipitation with sodium sulfide (Lahey et al., '52). The animals were given reduced iron, free of copper, in an amount more than sufficient to prevent the development of iron deficiency. They were fed from stainless steel troughs and were housed in galvanized iron pens.

In such animals growth was good. The animals remained fairly tidy and no change in the color of their hair was observed. They were active until severe anemia developed. In a few, however, the legs became deformed, ultimately to the point where the animals would neither stand nor walk. Large subcutaneous nodules developed on the points of pressure.

Anemia developed rapidly and was hypochromic and microcytic in type (table 5). The morphology of the anemia differed in no way from that of iron deficiency. When copper and iron deficiency were combined, the microcytosis and hypochromia as well as the degree of anemia were even more marked than when either deficiency was produced alone. Striking normoblastic hyperplasia of the red cells in the bone marrow accompanied these anemias.

In copper deficiency, a moderate leukopenia was observed (table 6). This does not occur in iron deficiency. On the other hand, the platelet count remained normal, whereas in iron deficiency a marked increase in platelets may be observed.

As would be expected, the plasma copper decreases in copper deficiency and the red cell copper falls as well (table 7). The mean corpuscular copper decreased to 26 micromicromicrograms, as compared with the normal value of 61. There was also a decrease in the concentration of copper in the

TABLE 5

Morphologic characteristics of the anemia

GROUP	NO. OF PIGS	AGE IN DAYS	V.P.R.C.[1]	M.C.V.	M.C.H.C.	BONE MARROW L: E
			ml/100 ml	μ^3	%	
Control	10	84	42	56	33	1.8
Copper-deficient	30	84	22	39	29	0.6
Iron-deficient	10	59	21	36	28	0.8
Copper- and iron-deficient	4	59	13	30	26	0.2

[1] V.P.R.C. = volume of packed red cells.
M.C.V. = mean corpuscular volume.
M.C.H.C. = mean corpuscular hemoglobin concentration.
L: E: = ratio of leukocytes to erythrocytes.

TABLE 6

Alterations in leukocytes and platelets

GROUP	NO. OF PIGS	W.B.C.[1]	P.M.N.	M.N.C.	PLATELETS
		$10^3/mm^3$	$10^3/mm^3$	$10^3/mm^3$	$10^3/mm^3$
Control	10	15.3	4.7	10.6	520
Copper-deficient	30	10.4	2.4	8.0	615
Iron-deficient	10	16.6	3.7	13.0	1,311
Copper- and iron-deficient	4	17.6	1.7	15.9	815

[1] W.B.C. = white blood cells.
P.M.N. = polymorphonuclear leukocytes.
M.N.C. = mononuclear leukocytes.

red cells. Of great interest is the fact that in copper deficiency the plasma iron decreases to the same degree as occurs in iron deficiency. The iron-binding capacity of the serum increases, although not to the same extent as in iron deficiency. The per cent saturation of the iron-binding protein is reduced greatly below normal. In combined copper and iron deficiency the decrease in red cell copper is particularly striking.

As would be expected, there is a marked reduction in the amount of copper in the livers of copper-deficient swine. In

TABLE 7

Chemical alterations

GROUP	NO. OF PIGS	RBC COPPER	PLASMA COPPER	PLASMA IRON	IRON-BINDING CAPACITY	
					Total	
		μg %	*μg %*	*μg %*	*μg %*	*% saturation*
Control	10	110	186	175	511	36
Copper-deficient	30	67	15	38	628	7
Iron-deficient	10	110	207	30	864	4
Copper- and iron-deficient	4	27	6	28	751	4

iron-deficient animals, there was a 4-fold increase in the copper content of the liver as compared with the normal, even though all of the animals were fed the same amount of dietary copper.

The pronounced morphologic changes accompanying copper deficiency are associated with no significant increase in free protoporphyrin in the red cells. There was a very slight increase in urinary coproporphyrin excretion. There was no significant alteration in any of the plasma protein fractions and no alteration from the normal in serum bilirubin content or in the fragility of the red corpuscles.

When copper sulfate is given to a copper-deficient animal, a sharp reticulocyte response occurs, there is rapid blood regeneration, the microcytosis disappears quickly and the hypochromia is corrected more slowly (fig. 6). The leuko-

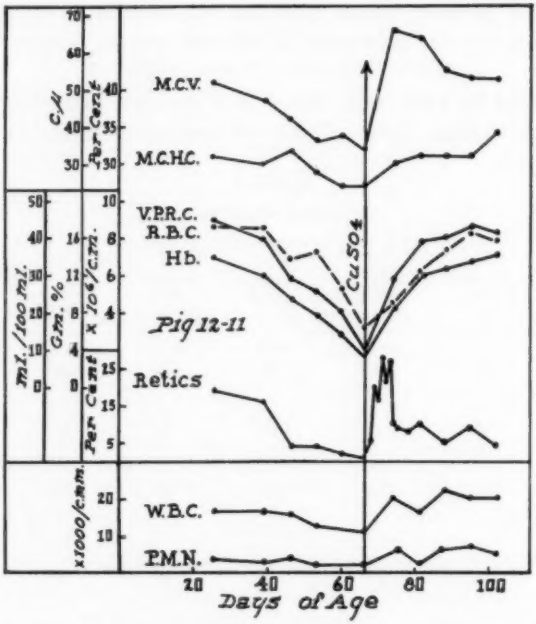

Fig. 6 Showing the development of microcytic, hypochromic anemia, leukopenia and neutropenia in a pig deficient in copper and the response of the blood to the oral administration of 0.5 mg of copper/kg of body weight/day.

M.C.V., mean corpuscular volume; M.C.H.C., mean corpuscular hemoglobin concentration; V.P.R.C., volume of packed red cells; R.B.C., red blood cells; Hb hemoglobin; W.B.C., total leukocytes; P.M.N., polymorphonuclear leukocytes.

cyte count rises to normal and the bone marrow resumes its normal appearance. The plasma copper increases rapidly, as does the plasma iron. The reduced red cell copper is corrected more slowly. The iron-binding capacity decreases rapidly, while the per cent saturation increases more slowly.

In animals in which a combined deficiency of copper and iron was produced it was found that when iron was given first, no response occurred (fig. 7). If copper was given first, only a slight reticulocyte response was observed. Substantial im-

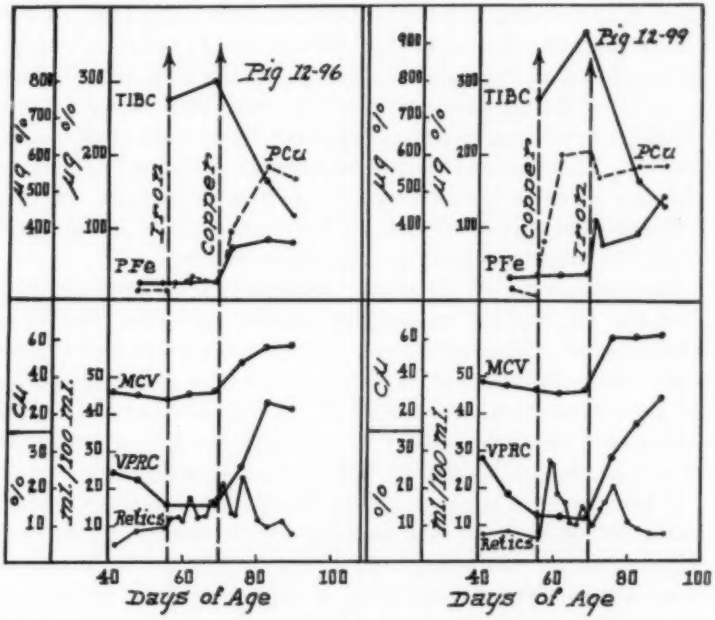

Fig. 7 Showing the response of two pigs deficient in both copper and iron to the oral administration of copper and iron.

Pig 12–96 was given iron (30 mg/kg of body weight/day), and 14 days later copper (0.5 mg/kg of body weight/day) was added to the diet. Pig 12–99 was first given copper (same amount as above) and then 14 days later, iron (amount as above) was added to the diet.

The administration of iron alone (pig 12–96) failed to increase the plasma iron level and no significant hemopoietic response occurred. The administration of copper alone (pig 12–99) was followed by a rapid increase in the plasma copper level and reticulocytosis but no alteration in the V.P.R.C. or other determinations took place. When both elements were added to the diet there was a prompt return to normal in all of the values.

T.I.B.C., total iron-binding capacity of the plasma; PFe, plasma iron; PCu, plasma copper; M.C.V., mean corpuscular volume; V.P.R.C., volume of packed red cells.

provement did not take place in either case until the second missing substance was furnished. Thus the lack of either copper or iron inhibited a hemopoietic response.

The striking similarity in the effects of copper and iron deficiency suggests that these two substances are closely related in their roles in hemopoiesis. In both experimental conditions the anemia is of the microcytic hypochromic type, there is hypoferremia and an increase in the total iron-binding capacity of the plasma, and the bone marrow is hyperplastic. The free erythrocyte protoporphyrin is normal. Differences are few. There is a moderate leukopenia in copper deficiency, whereas there is thrombocytosis in iron deficiency. In iron deficiency the copper content of the erythrocytes is unaltered, while the plasma copper is slightly increased.

The manner in which copper influences iron metabolism is obscure. In seeking an answer to this question, our work (Gubler et al., '52; Chase et al, '52a) has been directed toward study of the influence of copper on: (1) iron absorption from the gastrointestinal tract; and (2) the utilization of iron for hemoglobin synthesis either by (a) mobilization of iron from stores, or (b) utilization of iron in the bone marrow.

When the iron content of the blood, liver, kidneys, spleen and heart of viviperfused control, copper-deficient and iron-deficient swine was measured, it was found that there was as great a depletion of tissue iron in copper-deficient animals as in iron-deficient animals (table 8). The tissues of control animals contained almost 4 times as much iron as those of copper-deficient animals fed the same amounts of iron.

Again, when radioactive iron was given orally to control and to copper-deficient animals, as much as 6% was found in the tissues of the control animals, whereas only 2% was present in the tissues of the copper-deficient pigs. These observations suggest that in the absence of copper the absorption of iron is impaired.

The influence of copper on iron absorption is further indicated by observations on animals deficient in copper but fed ample amounts of iron (fig. 8). When the animals were

severely anemic as the result of copper deficiency, iron was withdrawn from the diet for a period of 5 days. Copper was then administered. There was a slight reticulocyte response but no blood regeneration. No more copper was given, but

TABLE 8

Iron analyses

TISSUE	CONTROL	COPPER-DEFICIENT	IRON-DEFICIENT
	mg Fe	*mg Fe*	*mg Fe*
Blood	518	130	193
Liver	46	18	7
Kidney	6	2	1
Spleen	12	8	3
Heart	3	3	2
Total	585	161	206
Body wt. (kg)	11	12	15
Iron/kg (mg)	53	13	14

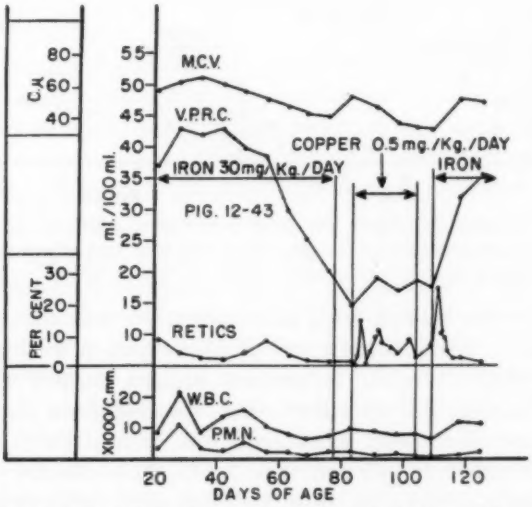

Fig. 8 Showing the failure of a copper-deficient pig to respond hemopoietically to copper after iron had been withdrawn from the diet for 5 days prior to giving the copper. After administering and then discontinuing the copper, the anemia was alleviated by the administration of iron.

after a 5-day interval, iron was administered. This was followed not only by a reticulocyte response but by rapid erythropoiesis. It would seem that once the tissues were supplied with copper, iron was absorbed.

The changes in plasma iron in animals deficient both in iron and copper illustrate this point well (fig. 9). When first iron was given orally, the plasma iron level remained un-

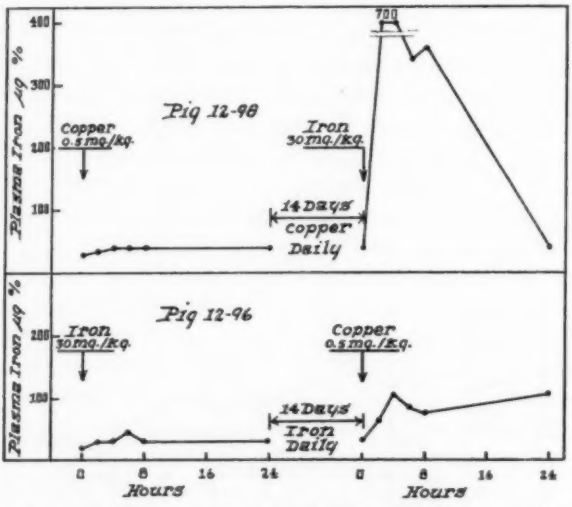

Fig. 9 Showing the plasma iron values immediately following oral therapy in two animals initially deficient in both copper and iron. One animal (12–98) was treated first with copper and the other (12–96) first with iron.

changed even though iron administration was continued for two weeks. When copper was administered, a modest rise in plasma iron followed. In another animal, copper was given orally for a similar length of time. No change in plasma iron took place. However, when iron was administered orally, a sharp increase in plasma iron took place. In this animal, whose tissues were by now well supplied with copper, the iron in the gastrointestinal tract was absorbed very rapidly.

Other experiments indicate, however, that failure of iron absorption is not the only defect in copper-deficient animals.

To circumvent the role of the bowel in the absorption of iron, copper-deficient animals were given iron ascorbate intraperitoneally. In such animals the development of anemia was not prevented, delayed or modified in any way. Other animals, also deficient in copper, were given colloidal iron intravenously. The amounts of iron administered were sufficient to eliminate the hypoferremia and could have relieved the anemia. However, no significant influence on the anemia was observed.

Several groups of animals were given radioactive iron intravenously. In the control animals 93% of the Fe^{59} appeared in hemoglobin within 6 days and in iron-deficient animals 83% was found in the hemoglobin. In the copper-deficient pigs, on the other hand, only 20 to 25% appeared in the hemoglobin. Thus it is evident that even when iron is supplied intraperitoneally or intravenously, the tissues of copper-deficient animals are unable to utilize iron for hemoglobin formation.

That, in the presence of copper, iron is mobilized from the tissues is indicated by another experiment (fig. 10). One group of copper-deficient pigs (A) were given only iron by mouth. The plasma iron level scarcely changed. Another group (B) were given iron orally together with copper. A sharp increase in the plasma iron level took place. The third group (C) were given no iron for 5 days and were then given copper only. The plasma iron increase was just as large as in the animals fed iron together with copper. Thus it appears that, even when iron is not present in the gastrointestinal tract, the administration of copper is followed by a rise in the plasma iron. One must conclude that such iron is coming from the tissues.

These studies, together with other similar ones which cannot be described here, suggest to us that copper favors iron absorption in the gastrointestinal tract, facilitates mobilization of iron from the tissues and is concerned in the utilization of iron in hemoglobin synthesis. Copper, in short, in-

fluences iron metabolism at the level of the mucosal cell and the liver cell and in the bone marrow.

Just how copper functions at the cellular level is an intriguing question. The earlier suggestion that copper might be essential for the synthesis of protoporphyrin is not borne out by our finding of normal free erythrocyte protoporphyrin in copper deficiency. We have been unable to obtain evidence that copper influences the rate of turnover of iron through

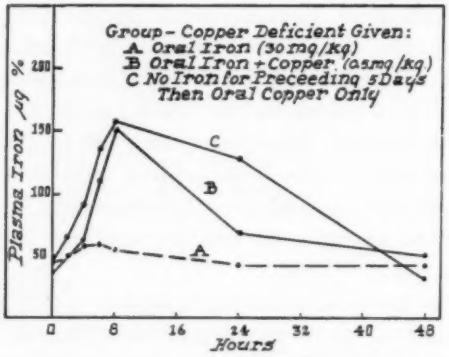

Fig. 10 Plasma iron levels immediately following the administration of iron (group A), iron and copper (group B), and copper only (group C) to copper-deficient swine. Curves A and C represent the mean value for two animals each and curve B represents the mean of 8 animals.

ferritin (Chase et al., '52b). As to the possibility that copper is essential for the incorporation of iron into certain enzymes, no answer can be given. However, we have not (Lahey et al., '52) been able to confirm the conclusion of Schultze ('39) that catalase activity is decreased in copper deficiency. We have no data concerning the reported (Schultze, '39) decrease in cytochrome oxidase activity in copper deficiency.

SUMMARY

Copper is found in blood in the plasma and in the cells. Red cell copper tends to be constant. The plasma copper,

on the other hand, increases under a great variety of circumstances. Hypocupremia is rare.

By far the greatest proportion of plasma copper is in the form of a copper protein corresponding to an α-globulin. A very small part of plasma copper is bound more loosely to one of several possible protein fractions and may represent copper in transit.

Deficiency of copper is associated with the development of severe, hypochromic microcytic anemia, hypoferremia as well as hypocupremia, moderate leukopenia and bone marrow normoblastic hyperplasia.

In copper-deficient swine fed ample amounts of iron, the total body content of iron, as measured by chemical assay of the tissues or by measurement of radioactive iron, is low as compared with normal. Copper therapy without the simultaneous administration of iron in such animals is ineffective.

In swine made deficient both in copper and iron, no increase in plasma iron follows iron therapy but an increase takes place promptly when copper is given as well. These observations are interpreted as indicating that copper favors iron absorption from the gastrointestinal tract.

That copper is also concerned in the mobilization of iron from the tissues is suggested by several observations. Thus, analysis of the iron content of copper-deficient animals reveals that the chief depletion of total body iron is in the hemoglobin compartment. This suggests that movement of iron from the tissues to the plasma is decreased. This view is also supported by the observation that in copper-deficient swine the administration of copper is followed by a very sharp rise in the plasma iron content.

That impaired utilization of iron for hemoglobin synthesis is another factor in the disturbed iron metabolism of copper deficiency is indicated by the fact that the intravenous or intraperitoneal administration of iron fails to relieve the anemia in copper-deficient animals. Furthermore, studies with intravenously-administered radioactive iron reveal that the uptake of iron for hemoglobin synthesis is poor in copper-deficient swine.

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SOME FACTORS AFFECTING THE GROWTH AND DEVELOPMENT OF RATS FED RANCID FAT

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Rancid fat provides an excellent tool for studying the nutritional requirements of animals under an adverse dietary condition. Diets which give optimum growth to animals when prepared with fresh fats have been found to be detrimental to growth or development when prepared with rancid fats. This adverse effect of rancidity may be due to one or more of the following factors: (1) a condition of stress induced by either an irritating effect or the presence of toxic agents; (2) interference with the nutritional property of the fat; (3) destruction of other nutrients; (4) altered intestinal function or flora; or (5) diminished food intake.

The literature on dietary components destroyed by rancid fats as well as on the effect of such diets on experimental animals has been reviewed by Burr and Barnes ('43), Quackenbush ('45), Barnes, Clausen, Rusoff, Hanson, Swendseid and Burr ('48) and French ('49). Dietary components listed by these reviewers as partially inactivated or destroyed by the inclusion of rancid fats in the diet are vitamin A and carotene, tocopherol, vitamin D, vitamin K, pantothenic acid, pyridoxine, biotin, ascorbic acid and the essential fatty acids.

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The present study was undertaken to determine the effect of rancid fat on the growth and development of the rat, apart from the induction of gross vitamin deficiencies. The supplemental factors used in the study were cortisone, antibacterial drugs, and increased protein in the diet.

EXPERIMENTAL

Male, weanling, Wistar-strain rats from our own breeding colony were used throughout the experiments. All animals were housed individually in raised wire-bottom cages and were supplied ad libitum with food and water. The weights of the animals were recorded weekly; the food consumptions of all animals were recorded at the time of feeding. All diets with fresh fat were fed twice weekly and all diets containing rancid fat were fed three times weekly, any food remaining in the cup at the time of feeding being discarded.

The basal diet consisted of 22% extracted casein (vitamin-low),[2] 60% sucrose, 4% salt mixture (U.S.P. XIV), 4% cellulose[3] and 10% fat (soybean oil).[4] Each kilogram of all diets had the following synthetic vitamins[5] incorporated in the basal mixture, replacing an equal weight of sucrose: 2,000 mg choline chloride, 1,500 mg inositol, 500 mg para-aminobenzoic acid, and 100 mg nicotinic acid. The remaining water-soluble vitamins given orally 5 times weekly were made up in a water suspension so that 0.05 ml contained 250 μg thiamine chloride, 500 μg each of riboflavin and pyridoxine, 1,200 μg calcium pantothenate, 300 μg folic acid, 5 μg biotin and 0.08 μg B_{12}.

The fat-soluble vitamins were injected intramuscularly in 0.05-ml doses twice weekly in alternate hind legs. Each 0.05-ml dose contained 500 μg 2-methyl 1,4 naphthoquinone, 500 I.U.

[2] Genatosan.

[3] Solka Floc, Brown and Co., New York, N. Y.

[4] Unilever Ltd.

[5] All synthetic vitamins, except B_{12}, were generously supplied by Roche Products Ltd., and by the Hoffmann-La Roche Co., Basle, Switzerland. Vitamin B_{12} was kindly supplied by Dr. Lester Smith, Glaxo Ltd., Greenford.

vitamin A acetate, 50 I.U. calciferol, 3.5 mg alpha-tocopherol (free) and 3.5 mg alpha-tocopherol acetate.

Diets were prepared in 1- or 2-kg lots, and these were stored at 2° ± 1°C. for not more than two weeks. To each batch of 100 gm of fat, fresh or rancid, was added 0.1 ml of ethyl laurate containing 500 μg diacetyl,[6] giving 5 parts per million of diacetyl in the fat. This was done in an attempt to overcome any aversion the rats might have for the rancid fat diets due to odor. Earlier work by Deuel and Movitt ('44) showed some beneficial effects on the food consumption of rats when diacetyl was used in certain diets. The rancid fat diet, in spite of the high peroxide value, smelled only slightly "off," but when the diacetyl was included it was almost impossible for humans to distinguish rancid from fresh diets by odor alone.

The rancid soybean oil was prepared by bubbling air through the oil, which was constantly heated in a water bath to a temperature of 50° ± 0.1°C. The soybean oil was rancidified in batches of 1,600 ml to a peroxide value of 530 to 550 μ moles O_2/gm, as determined by the method of Paschke and Wheeler ('44), and was stored at − 20°C. The rancid fat gave a strongly positive Kreis test and had an acid value of 0.21.

Changes in the diets will be described as the individual experiments are reported.

Several animals from each group were autopsied at the termination of the experiments. Animals to be autopsied were exsanguinated while under light ether anesthesia and organs were excised, trimmed and weighed immediately, except for the intestine, which was cut at the pylorus and at the ileocaecal junction, measured for length and then washed through three times with 20 ml each of distilled water. The small intestine was then laid out and opened lengthwise by scissors, and blotted lightly by touching once along the whole intestine with filter paper. The intestine, after having been weighed wet, was dried in a freeze drier and reweighed for dry weight.

[6] Hopkins and Williams Ltd.

The determination of fecal fat was made on 7-day collections from individual rats during the final week of the 10-week experiment. Samples were dried at 100°C. for 24 hours and weighed, then crushed and extracted by a method used by H. G. Sammons (personal communication) in which the 7-day fecal collection from each rat is refluxed with 30 ml 1N HCl for 30 minutes. The acid is then decanted and the moist feces are washed once with 95% alcohol, which in turn is decanted also. Then the feces are extracted twice by refluxing 30 minutes each time with 30 ml of chloroform. All extracts are combined and dried on the water bath; the fat is taken up in ethyl ether, dried over anhydrous sodium sulfate and weighed after drying. Proteolytic activity of the intestinal washings was determined by the method of Charney and Tomarelli ('47) and is called "trypsin value" in this report.

RESULTS

A summary of the data concerning growth and food consumption and the relative values of several supplements for growth is presented in table 1. All animals in the 14 experimental groups were fed the 10% fresh oil diet (F-1) for 5 days previous to the experimental period, in an attempt to give the animals sufficient essential fatty acids so that this factor might not be a limiting one in the later growth of the animals on rancid fat.

Food consumption

The total food eaten by rats fed rancid fat was less than that by those fed fresh fat (table 1). However, the data for weight gain (grams) per 100 gm of food eaten reveal that the "efficiency" of food utilization (assuming the rancid fat has the same caloric value as the fresh fat) was just as good during the last 7 weeks of the experiment for the rancid fat groups as for the fresh fat groups. In view of the good "efficiency" of utilization of the rancid fat diets during the last 7 weeks, the failure of growth of these animals during

TABLE 1

The influence of cortisone, whole liver, drugs and increased protein on the growth and food consumption of rats fed 10% fresh or 10% rancid soybean oil

GROUP NO.	SUPPLEMENT	BODY WEIGHT		WEIGHT GAIN AT END OF FOLLOWING WEEKS [3]			WT. GAIN, LAST 7 WKS.	FOOD EATEN PER DAY		WT. GAIN /100 GM FOOD EATEN	
		Initial [1]	After 5 days [2]	3	7	10 [4]		0–3 wk.	3–10 wk.	0–3 wk.	3–10 wk.
		gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
					FRESH FAT						
F-1	None	51	68	85 (6)	172 (6)	198 ± 20 (6)	115	10.0	12.0	40.6	19.5
F-2	Cortisone [5]	53	60	80	147	171 ± 7	91	8.9	11.4	42.9	16.2
F-3	Drugs [6]	49	66	81 (7)	180 (7)	204 ± 11 (7)	123	9.7	12.6	39.5	19.9
F-4	Drugs [6] and cortisone [5]	51	56	72	147	167 ± 8	95	9.4	11.6	36.5	16.7
F-5	10% whole liver [7]	48	63	82	172	196 ± 7 (7)	114	11.2	16.3	35.4	14.3
F-6	10% whole liver [7] + drugs [8]	52	69	84	156	183 ± 10	99	11.3	14.8	35.4	13.6
F-7	8% added casein [9]	51	66	85	168	193 ± 9	108	10.8	13.6	35.9	16.2
					RANCID FAT						
R-1	None	52	68	16	80	105 ± 5 (7) [10]	89	6.2	9.7	12.3	18.8
R-2	Cortisone [5]	53	60	17	72	94 ± 9	77	6.2	8.6	13.1	18.2
R-3	Drugs [6]	52	71	−8	46	80 ± 9	88	4.4	8.0	0	22.3
R-4	Drugs [6] + cortisone [5]	50	58	1	48 (6)	68 ± 8 (6)	67	5.2	8.1	0.9	16.8
R-5	10% whole liver [7]	53	69	21	99 (6)	122 ± 8 (6)	101	7.5	13.4	13.4	15.4
R-6	10% whole liver [7] + drugs [8]	53	68	13 (7)	79 (7)	119 ± 9 (7)	106	6.6	12.7	9.3	17.0
R-7	8% added casein [9]	55	74	46	123	151 ± 8	105	7.8	11.9	28.0	18.0

[1] Eight male rats per group.

[2] Rats on all groups fed basal fresh fat diet (F-1) for 5 days. Weight after 5 days used as initial weight for determinations of weight gain in this experiment.

[3] Figures in parentheses are given when rats have died, and represent the number alive at the times indicated.

[4] Includes the standard error of the mean as determined by the formula $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$, where "d" is the deviation from the mean and "n" is the number of observations.

[5] "Cortone" (Merck), 25 mg/ml. Diluted with physiological saline and injected in a 0.1-ml volume subcutaneously daily (6 days a week) from the time the animals were first put on the 5-day pre-feeding period. During the 5-day pre-feeding period, and for 21 days thereafter, the rats were given 0.25 mg cortisone per day. For the next 14 days, until the 35th day of the experimental period, they were injected with 0.40 mg cortisone per day, and from the 35th day until the termination of the experiment, they were given 0.625 mg cortisone per day.

[6] Drugs added to the diet in place of an equal weight of sucrose were 1% phthalylsulfathiazole (May and Baker Ltd.) plus 0.01% chloromycetin (Parke, Davis Ltd., Hounslow) for the first 21 days of the experimental period, when the diet was changed for the remainder of the experiment to include 0.02% neomycin sulfate (Upjohn) instead of the aforementioned drugs.

[7] Desiccated whole liver (Payne and Byrne, Greenford).

[8] Same levels of phthalylsulfathiazole and chloromycetin as used in group F-3 but continued without change throughout the experimental period.

[9] Diet increased by 8% vitamin-low casein in place of sucrose to make 30% casein in the diet.

[10] Animal killed by accident.

the first three weeks was probably a reflection of lowered food consumption rather than of a lowered nutritive value for the rancid fat diets. An analysis of the growth data by the log weight-reciprocal time curves of Zucker and Zucker ('42) substantiates these conclusions.

Treatment with cortisone [7]

Cortisone was chosen as a factor which might be expected to aid an animal suffering from a non-specific stress if that were the cause of the failure of rats fed rancid fat to grow normally. An examination of the data shows that at the dosage levels of cortisone used there was no beneficial effect on the growth of the rats fed rancid fat, and that actually the cortisone treatment of the rats fed rancid fat acted to depress growth in a manner similar to that observed with the rats fed fresh fat.

Treatment with anti-bacterial drugs

The growth data for rats given 1% phthalylsulfathiazole plus 0.01% chloromycetin [8] in the diet for the first 21 days and 0.02% neomycin sulfate [9] for the remainder of the experimental period are presented in groups F-3 for the fresh fat- and R-3 for the rancid fat-supplemented animals (table 1). The mixture of sulfa drug plus chloromycetin had a severely depressive effect on the growth of the rats given rancid fat (R-3) but no apparent effect on those receiving fresh fat (F-3). The neomycin sulfate seemed to have neither harmful nor beneficial effects on the growth of the rats at the level it was fed, and during the period it was included in the diet (last 7 weeks).

[7] The authors are indebted to Dr. E. Alpert, Clinical Research Laboratories, Merck, Inc., Rahway, N. J., for the "Cortone" used in this work. See table 1, footnote 5, for schedule of administration.

[8] Synthetic chloramphenicol (chloromycetin) was generously supplied by Dr. R. Hodgkinson, Parke, Davis and Co. Ltd., Hounslow.

[9] Neomycin sulfate kindly supplied by Dr. J. S. Evans of Upjohn and Co., Kalamazoo, Mich.

Treatment with liver

Desiccated whole liver, when added to the diet at a level of 10% in place of an equal weight of sucrose, had some beneficial effect when used in the rancid fat diet (group R-5, table 1), but no effect on growth of the animals fed the fresh fat diet (F-5). The difference in over-all growth between group R-1 (105 gm gain) and R-5 (122 gm gain) is not significant ($P > 0.05$); however, this difference was constant throughout the experimental period. When 1.0% phthalylsulfathiazole + 0.01% chloromycetin, as used for the first 21 days with groups F-3 and F-4, R-3 and R-4, were given throughout the 10-week experimental period to the liver groups (F-6 and R-6), they were without any adverse effect on the growth of the rats.

A comparison of weight gains during the last 7 weeks reveals equal or superior growth for the animals on rancid fat receiving 10% whole liver and 1.0% phthalylsulfathiazole plus 0.01% chloromycetin (group R-6) compared with that of animals on the fresh fat diet (F-6) and receiving the same supplements. Also, during this period the "efficiency" of the diet for growth was markedly greater (17.0) for the rancid fat group R-6 than for the fresh fat group F-6 (13.6).

Increased casein in diet

An increase of casein in the diet from 22% (groups F-1 and R-1) to 30% (groups F-7 and R-7) had no beneficial effect on the growth of the animals receiving fresh fat but had a highly significant beneficial influence on the growth of the animals fed rancid fat. The 10-week weight gain for the 30% casein diet was 151 gm, while that for the 22% casein diet was 105 gm ($P < 0.001$). The greatly increased ratio of weight gain to food eaten in the first three weeks of the experiment, when the 30% casein level (ratio 28.0 for group R-7) is compared with the 22% casein level in the diet (ratio 12.3 for group R-1), is also striking.

Since it was indicated in experiment 1 that the limiting factor in the growth of rats fed rancid fat might be the protein level of the diet, a second experiment was performed to investigate this possibility. Twenty-two per cent casein in the diet had been found to give optimum growth with fresh fat in experiment 1; therefore this same level (22%) was used along with lower levels of casein (16 and 12%) in the fresh fat diets in experiment 2. Levels of 22, 30 and 38% casein in the diet were fed with rancid fat. Growth data, food consumptions, and the "efficiencies" of the diets are presented in table 2. Both weight gain and "efficiency" of food utilization were affected by decreasing the protein level from 22 to 16% or to 12% in the fresh fat diets. Twenty-two per cent protein in experiment 2, as in experiment 1, gave inferior growth when fed with rancid fat; whereas 30% protein in the rancid fat diet (R-11) increased growth and "efficiency" significantly over that obtained with the 22% level. A further increase to 38% protein (R-12) in the rancid fat diet provided no greater measurable improvement in growth. The difference between the weight gains for the 30% casein diet with rancid fat (R-11) and those for the 22% casein diet with fresh fat (F-8) was not significant ($P > 0.05$). The "efficiency" data reveal that at 30 or 38% protein in the diet the utilization of food for growth was the same as for the 22% protein control group with fresh fat. The difference in growth between F-8 on the one hand and R-11 or R-12 on the other would therefore appear to be related to differences in food intake.

Physical appearance of animals

Diarrhea was not seen in all animals on rancid fat; in some it appeared sporadically, while in others it persisted throughout the experiment. The feces of the two liver groups receiving rancid fats (R-5 and R-6) were always soft and of much greater bulk than those of any of the other groups. The animals of group R-7, receiving 30% protein in the diet, showed little diarrhea during the experimental period. Priapism was

TABLE 2

Effect of varying protein content of the diet on the growth and food consumption of rats fed 10% fresh or 10% rancid soybean oil
("F" represents fresh fat diets and "R" represents rancid fat diets)

GROUP NO.	CASEIN IN DIET	BODY WEIGHT			WEIGHT GAIN [4] (4 WKS.)	FOOD EATEN /DAY [5]	WT. GAIN /100 GM FOOD EATEN [6]
		Initial [1]	After 5 days [2]	After 14 days [3]			
	%	gm	gm	gm	gm	gm	gm
			F	R	F	F	F
F-8	22	55.2	64.8	69.3	116.7 ± 6.7	11.6	35.9
F-9	16	56.2	64.2	68.3	100.8 ± 8.1	12.3	29.3
F-10	12	55.2	67.2	72.2 (8)	78.0 ± 8.2 (8)	11.8	24.9
			F	R	R	R	R
R-8	22	55.6	69.0	68.6	57.0 ± 4.2 (8)	8.1	25.2
R-11	30	54.0	67.7	68.6	96.6 ± 7.0	10.4	33.1
R-12	38	54.6	67.4	67.2	98.0 ± 7.4	10.1	34.8

[1] Nine male rats per group. Figures in parentheses represent animals alive at the time indicated and therefore the number of rats averaged in the group.

[2] Rats on all groups fed fresh fat diet F-8 for 5 days as in table 1.

[3] Rats on all groups fed rancid fat diet R-8 from the 6th day through the 14th day, and the average weight at the end of this period is used as the initial weight for calculations of weight gain during the experimental period.

[4] Includes standard error of the mean; see footnote 4, table 1.

[5] Expresses value for food consumed during 4-week experimental period only.

[6] Calculated from 4-week experimental weight gain and from 4-week experimental food consumptions only.

observed in groups R-3 (two animals, 25%) and R-4 (4 animals, 50%). One case of priapism occurred before the change to neomycin sulfate, while 5 cases occurred after the change. Other reports of priapism in rats have been made for such diverse conditions as biotin deficiency (Deuel and Greenberg, '47), high cortisone dosage (Ershoff, '52) and subsistence on mineral oil diets (Bacon et al., '52).

The rats on the second experiment (table 2) showed no priapism during the 4 weeks on the experiment, little diarrhea on the 22% casein diet (R-8) and no diarrhea on the 30% casein diet (R-11). However, the rats of group R-12 (38% casein) had severe diarrhea with the passage of much gelatinous-like material. The loose feces were associated with excoriation of the anal region, a condition not seen in any other experimental group.

The animals on the 30% casein diets R-7 and R-11, when grouped with rats on the fresh fat diets, could not be identified as abnormal animals except by ear punches.

Effect of rancid fat on organ weights of experimental rats

The average weights of testes, seminal vesicles, liver, kidneys, adrenals, thymus, heart (ventricle) and spleen for control fresh fat groups and for rancid fat groups were determined at autopsy and are presented in table 3.

The testes grew to normal size in all rancid fat groups even though body size was restricted in these groups. As a consequence, the ratio of testes to body size was much larger for these animals than for those on the fresh fat diets. These results are consistent with data published by Jackson ('15, '32) for rats with restricted growth. The seminal vesicles of the rats on rancid fats were all well-developed, indicating that these testes were producing androgenic hormones. All other organs listed in table 3 appeared to have been little affected in size by the feeding of rancid fat. Liver supplementation caused a marked increase in the absolute and rela-

TABLE 3

Experiment 1. Effects of rancid fat on organ weights of rats on diets supplemented by cortisone, whole liver, drugs and increased protein. Control groups received fresh fat

(Organ weights per 100 gm body weight presented in parentheses)

GROUP NO.	SUPPLEMENT [1]	BODY WT., END 10 WKS.[2] ON EXP.	TESTES	SEMINAL VESICLES	LIVER	KIDNEYS	ADRENALS	THYMUS	HEART (Ventricle)	SPLEEN
		gm	*gm*	*mg*	*gm*	*gm*	*mg*	*mg*	*mg*	*mg*
					FRESH FAT					
F-1	None	272 (3)	2.514 (0.934)	1045 (393)	8.90 (3.32)	1.650 (0.613)	39.2 (14.6)	241 (88)	642 (238)	791 (297)
F-2	Cortisone	248 (3)	2.666 (1.073)	817 (335)	8.53 (3.44)	1.599 (0.644)	33.9 (13.7)	186 (75)	641 (259)	695 (280)
F-5	10% Whole liver	262 (3)	2.743 (1.047)	752 (286)	10.64 (4.07)	2.437 (0.932)	67.6 (26.0)	267 (101)	990 (380)	1436 (543)
F-6	10% Whole liver + drugs	238 (3)	2.548 (1.078)	617 (264)	10.26 (4.33)	2.055 (0.868)	62.5 (26.3)	253 (108)	854 (359)	1100 (458)
F-7	8% Added casein	280 (3)	2.696 (0.954)	766 (262)	10.43 (3.70)	1.984 (0.709)	42.9 (15.3)	360 (129)	721 (258)	901 (320)
					RANCID FAT					
R-1	None	173 (4)	2.494 (1.437)	684 (391)	7.63 (4.42)	1.414 (0.822)	29.6 (17.2)	216 (125)	535 (310)	503 (287)
R-2	Cortisone	160 (3)	2.600 (1.629)	580 (361)	7.69 (4.80)	1.407 (0.877)	24.7 (15.4)	163 (102)	519 (324)	493 (307)
R-5	10% Whole liver	178 (2)	2.509 (1.419)	460 (258)	10.10 (5.56)	2.132 (1.180)	33.8 (19.0)	170 (96)	776 (430)	928 (519)
R-6	10% Whole liver + drugs	198 (4)	2.711 (1.372)	553 (280)	10.58 (5.35)	1.931 (0.978)	40.6 (20.6)	228 (113)	806 (408)	740 (374)
R-7	8% Added casein	233 (3)	2.634 (1.134)	1008 (434)	9.27 (3.95)	1.642 (0.704)	34.4 (14.7)	220 (96)	653 (279)	719 (310)

[1] See table 1 for details of supplementation.
[2] Figures in parentheses represent number of rats autopsied.

tive weights of liver, kidneys, heart, adrenals and spleen in animals on both the fresh and rancid fat diets (groups F-5 and F-6 and R-5 and R-6). The cause of the marked increase in weight of these organs when liver is fed is not readily apparent. When cortisone was given to groups F-2 and R-2, it caused a small but consistent reduction in the size of the adrenals, the thymus and the spleen, as had been expected.

The data concerning weights of the organs of the alimentary tract, as well as the moisture contents and lengths of the small intestines of the experimental animals, are tabulated in table 4. Although the ratio of stomach weight to body weight was increased when rancid fats were fed, in no case was the absolute stomach weight greater for rancid fat groups than for similar fresh fat groups. The major effect of rancid fat on the organs examined would appear to have been on the intestinal tract, since the wet weights of the small intestines were significantly greater for most groups fed rancid fat than for similar groups fed fresh fat.

The dry weights of the small intestines were also greater for the rancid fat groups than for the fresh fat groups, except in the R-3 and F-3 groups, where they were the same.

Comparatively few rats were used for the intestinal weight study in each group; therefore, the statistical evaluation of probability for each group may not give a true picture of the results. Since there was a consistently greater weight of wet intestines for each rancid fat group when compared to the corresponding fat control group, it may be more appropriate to compare the entire number of samples (19) from the fresh fat groups with those (21) from the rancid fat groups. The average wet weight of the intestines of the fresh fat group was 4.79 ± 0.18 gm, while that of the rancid fat groups was 6.51 ± 0.25 gm. Using the "t" test, the probability of significance of this difference is found to be < 0.001. Although the absolute dry weights of the intestines of each rancid fat group were not significantly larger than those of the corresponding fresh fat group, the same technique was used to determine the probability of significant difference between the

TABLE 4

Experiment 1. Effect of rancid fat

TABLE 4

Experiment 1. Effect of rancid fat on alimentary tract organ weights and on moisture content and length of small intestine. Supplemented by cortisone, whole liver, drugs and increased protein. Control groups received fresh fat

GROUP NO.	SUPPLEMENT [1]	BODY WT.,[2] END 10 WKS. ON EXP.	STOMACH WT.	STOMACH WT., MG/ 100 GM BODY WT.	SMALL INTESTINE							
					Wet wt.[3]	"P" [4]	Gm/ 100 gm body wt.	Dry wt.[3]	"P" [4]	Gm/ 100 gm body wt.	Moisture	Length
		gm	*mg*		*gm*			*gm*			%	*cm*
					FRESH FAT							
F-1	None	272 (3)	878	3.24	4.68 ± .24		1.72	0.88 ± .08		0.32	81.16	94.4
F-2	Cortisone	248 (3)	959	3.87	4.14 ± .23		1.67	0.81 ± .08		0.32	80.57	95.3
F-3	Drugs	308 (2)	1,000	3.26	4.14 ± .14		1.36	1.09 ± .21		0.36	79.97	100.4
F-4	Drugs and cortisone	228 (3)	919	4.03	4.31 ± .21		1.89	0.76 ± .00		0.34	82.16	85.8
F-5	10% Whole liver	262 (3)	1,021	3.90	5.92 ± .25		2.21	1.09 ± .06		0.42	81.62	101.3
F-6	10% Whole liver + drugs	238 (3)	930	3.91	5.35 ± .29		2.23	0.97 ± .11		0.40	82.06	102.0
F-7	8% Added casein	280 (3)	981	3.51	4.94 ± .44		1.76	1.01 ± .10		0.36	79.50	98.2
					RANCID FAT							
R-1	None	173 (4)	818	4.73	6.47 ± .56	0.05	3.74	1.18 ± .13	N.S.	0.68	81.94	99.8
R-2	Cortisone	160 (3)	820	5.11	6.24 ± .16	0.01	3.89	1.03 ± .12	N.S.	0.65	83.34	103.5
R-3	Drugs	137 (2)	898	6.56	5.93 ± .32	0.02	4.33	1.07 ± .16	N.S.	0.78	81.98	99.0
R-4	Drugs and cortisone	118 (3)	725	6.18	5.47 ± .60	N.S.	4.64	0.89 ± .10	N.S.	0.76	83.57	93.1
R-5	10% Whole liver	178 (2)	757	4.24	8.02 ± .74	0.05	4.52	1.43 ± .19	N.S.	0.80	82.29	111.2
R-6	10% Whole liver + drugs	198 (4)	880	4.46	7.32 ± .64	N.S.	3.72	1.28 ± .10	N.S.	0.65	82.47	103.9
R-7	8% Added casein	233 (3)	895	3.83	6.18 ± .59	N.S.	2.64	1.13 ± .06	N.S.	0.48	81.65	107.9

[1] See table 1 for details of supplementations.
[2] Figures in parentheses represent number of rats autopsied.
[3] Includes standard error of means; see footnote 4, table 1.
[4] Represents probability calculated from the "t" values when rancid fat group is compared with same dietary group receiving fresh fat.
"N.S." means not significant or a probability greater than 5 in 100.

two groups. The average dry weight of the intestines of the fresh fat groups was 0.93 ± 0.04 gm, while that of the rancid groups was 1.14 ± 0.05 gm. Using the "t" test, the probability of significance of the difference is found to be < 0.01.

The percentage moisture contents and lengths of the small intestines were consistently greater, although not to any significant degree, for all groups in experiment 1 receiving rancid fat, except for group R-3, than for comparable dietary groups receiving fresh fat (table 4).

Trypsin values were determined on the water washings from the entire small intestine for the unsupplemented 22% casein basal groups F-1 and R-1, the cortisone-treated groups F-2 and R-2, and the 30% casein groups F-7 and R-7. The values obtained were within the ranges usually found in the normal rat, indicating no impairment in proteolytic activity in the presence of rancid fat.

The organ weights of the rats on experiment 2, in which the level of casein was varied, were determined. The effect of rancid fat on the weights of these organs was similar to that observed in tables 3 and 4, experiment 1, and these weights are therefore not reported here.

Fecal fat analysis

Digestibilities (representing percentages of fat intakes absorbed) of the fats fed to three fresh fat groups (F-1, F-2 and F-7) and to the corresponding rancid fat groups (R-1, R-2 and R-7) were determined.

The digestibilities of the fresh fat ranged from 97.0 to 98.1% with the various diets fed. With the same diets the rancid fat digestibilities ranged from 92.8 to 95.0%, a lower range of percentages but still not abnormally low.

No correction was made for "metabolic fat" or fat excreted in the feces of the rats on a fat-free diet. If such a correction, which is considered unnecessary for this comparative study, had been made, the percentage of digestibilities of the fats eaten would have been higher.

DISCUSSION

Study of the food consumption data reveals that the interference with growth in the rancid fat group can be largely explained by the inadequate food intake of the rats. The low intake was marked for the first two to three weeks, during which time the growth of the animals was severely restricted, but at the end of this adjustment period the rats started to eat much more of the diet containing the rancid fats, and from this time onwards exhibited growth rates parallel to those of the control groups; their utilization of the food was normal, as is shown by the weight gain per 100 gm of food eaten (table 1).

The reason for this failure to eat during the first two to three weeks of the experiment is not apparent. The possibility that deficiencies played some part has to be considered. Destruction of the water-soluble vitamins was guarded against by feeding them separately and in massive dosage. If some destruction of the vitamins in the food mixture had occurred, it was not likely that a deficiency would have resulted. Furthermore, when this dosage was doubled in subsequent studies still in progress, no significant change in the response of the animals to the rancid fat was observed. All of the fat-soluble vitamins were given in large doses parenterally throughout the experiment. None of the animals showed any of the classical signs associated with deficiency of any of these vitamins.

It is unlikely that a deficiency of essential fatty acids existed in these experiments, since some linoleic acid probably remained unchanged in the oxidized oil and, furthermore, all the animals were given a preliminary period of feeding on fresh fat, which should have provided an adequate store of essential fatty acids for the period studied. The one dietary change which appeared to be effective in modifying the growth-inhibiting effect of the rancid fat was an increase of protein intake. This increase caused a significant decrease in the period of low food intake, so that when a 30% casein diet was

fed, normal growth began at about the 4th day, whereas with the lower protein level (22%) it did not begin until about the 16th day. Liver added to the diet had a similar effect on food consumption and on growth, which may have been due to its high protein content (64%). It should be noted, however, that the feeding of liver had effects on organ weights not attributable to protein alone.

This beneficial effect of protein may be due either to direct "neutralization" of a toxic factor in the rancid fat or to the relief of some dietary deficiency caused by the rancid fat. From consideration of the growth data it is not possible to decide between these possibilities. This increased requirement for protein in the rat when its diet contains rancid fat is contrary to the observations of Barnes et al. ('48), who failed to obtain improvement with increased dietary protein in animals fed a similar diet. It is possible, however, that their animals were suffering from concomitant folic acid and biotin deficiencies. Kaunitz and Slanetz ('50), and Kaunitz, Johnson and Slanetz ('52) in their studies of the effect of rancid fats on vitamin A and riboflavin requirements, concluded that rancid fat was not toxic per se. However, these workers used a 30% level of protein, so that the need for a higher than normal protein intake was not apparent. The peroxide level of the fat used in our experiments was about twice that used by Kaunitz et al., which may account for a few minor differences between their results and ours.

Organ weights

The second group of observations relates to changes in organ weights relative to body weight. Comparing the rancid fat group with the animals fed fresh fat, there were no significant differences in the weights of the heart ventricles, testes, seminal vesicles, livers or kidneys. No gross pathology was observed in either the livers or kidneys. The adrenals, thymus and spleen showed no significant differences.

Whether the rancid fat had elicited a non-specific stress reaction with the classical effects described by Selye ('51) is not answerable on the basis of the organ data, since the rats may have adapted themselves during the experimental period to such an extent that early effects had been overcome. Although no beneficial effects were discernible when cortisone was administered, improper dosage could have been a masking factor.

Under the conditions of this experiment rancid fat caused a significant increase in both the wet and dry weights of the small intestine. This increase in weight may have been due either to edema of the wall of the intestine or to increased mucus secretion, or both. Since cortisone failed to produce any beneficial effect in the animals receiving rancid fat, it seems likely that the major effect is one of increased mucus secretion. Attempts to differentiate between these two types of reaction by more direct methods have not been successful thus far. The action on the small intestine is similar to that produced by certain other low-grade irritants, such as long-chain and short-chain fatty acids and hypertonic solutions (Frazer, French and Thompson, '49). There is no reason to assume that the alteration in weight of the small intestine is necessarily associated with the growth-inhibiting action of the rancid fat.

Gastrointestinal studies

The third group of investigations carried out on these animals was concerned with gastrointestinal function. The digestive activities of the intestine, including pancreatic enzyme production, appeared to be the same in both groups of animals. The over-all absorption of fat was also within normal limits in both groups, although absorption seemed to be slightly less efficient in the rancid fat group. Since the fat digestibility data were obtained from feces gathered during the last week of the experiment, one cannot rule out the possibility that a defect in fat absorption could have existed

during the first few weeks. The administration of antibacterial drugs in these experiments caused uniform deterioration in the growth of the rats receiving rancid fats but had no effect on those fed fresh fat. It is clear, therefore, that the drug-sensitive organisms in the intestines of the animals receiving rancid fats were not contributing to the toxic action; it is possible that the intestinal organisms may have been making some contribution toward relief of secondarily induced dietary deficiency.

In conclusion, it is clear that there is an increased protein requirement for the rat when it is fed rancid fat with a high peroxide content under conditions that preclude the existence of vitamin deficiencies. This effect may be due to some direct damage to the nutritional value of the dietary protein. The only demonstrable effect that the diet containing rancid fat has on the rat fed adequate protein is an increase in the absolute and relative weights of the small intestine, possibly due to increased mucus secretion. There is no reason to suppose that this effect on the intestine contributes to the deleterious effect of the rancid fat under the conditions of these experiments.

SUMMARY

Rancid fat in the diet increases the protein requirement of the rat for optimum growth.

A diet containing 10% rancid soybean oil (peroxide no. 530–550 μ moles/gm) supported almost normal growth in rats when all known essential nutrients were supplied in large amounts and protein constituted 30% of the diet. The difference in growth between a group of rats fed rancid fat with an optimum level of all other known nutrients and a group fed an equivalent fresh fat diet can be accounted for in the main by the lowered consumption of the rancid fat diets during the first days of the experimental period.

The only organ weight seriously affected by the presence of the rancid fat was that of the intestinal tract.

Whole desiccated liver, although it had effects on organ weights not attributable to its protein content alone, appeared to benefit the rats in proportion to the protein content when added to the diet.

Cortisone had no beneficial effect on the growth of rats fed rancid fat under the experimental conditions employed.

Several drugs administered to modify intestinal flora had no beneficial effects on the growth of rats fed rancid fat.

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EFFECT OF DIETARY ANTIBIOTICS ON THE GROWTH OF CHICKS RECEIVING SUBOPTIMUM AMOUNTS OF THIAMINE BY MOUTH OR BY INJECTION [1, 2, 3, 4]

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Antibiotics stimulate the growth of chicks fed a wide variety of natural or synthetic diets, but how they act has not been established. Favored hypotheses are that antibiotics suppress intestinal microorganisms that either compete with the host for an essential nutrient or that cause low grade infections sufficient to diminish the growth of ordinary birds. Other possibilities are that organisms may be favored which synthesize the limiting nutrient, or that the antibiotic may act directly upon the host rather than upon its microflora (Elam et al., '51). Most of the growth responses observed do not permit the observer to distinguish between the various mechanisms possible, but when the growth response to an antibiotic is greater on a deficient diet than on a complete one, the operation of the mechanism of nutrient sparing is a strong pos-

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sibility. Such was the case in rat experiments by Linkswiler et al. ('51), Lih and Baumann ('51), and Sauberlich ('52), and in certain chick experiments by Biely and March ('51), and Coates et al. ('51). In the present study the effectiveness of antibiotics with respect to growth in chicks is shown to depend upon the method of administering the limiting nutrient.

EXPERIMENTAL

The chicks were the progeny of New Hampshire males and Single-Comb White Leghorn females fed diet B-1 of Robblee et al. ('48). Ten or more day-old chicks, 35 to 41 gm in weight, were used per group. They were kept in electrically heated batteries with raised screen floors, and were fed a basal diet of the following percentage composition: carbohydrate 61, hot alcohol-extracted casein 18, gelatin 10, salts V (Briggs et al., '43) 6, soybean oil 4, "feeding oil"[6] 0.5, DL-methionine 0.3, choline chloride 0.2, and inositol 0.1 gm per 100 gm of ration. The following were added, in milligrams per kilogram: biotin 0.2, menadione 0.5, α-tocopherol 3.0, folic acid 4.0, pyridoxine·HCl 4.0, riboflavin 6.0, calcium pantothenate 20, niacin 50, *p*-aminobenzoic acid 100, and vitamin B_{12} 0.030. The dextrin used was autoclaved corn starch. Levels of thiamine·HCl were added as indicated in the tables. The antibiotics procaine penicillin G, aureomycin·HCl, bacitracin and streptomycin were in all cases supplied in the diet.

The chicks in the injected groups received varying amounts of thiamine subcutaneously over the breast muscle, the amounts being chosen to permit growth at about the same rate as that of chicks fed suboptimum levels of thiamine. The experiments lasted from 11 to 28 days.

In certain experiments on the stability of thiamine in chick diets, glycerol replaced an equivalent portion of sucrose. The diets were stored in brown glass bottles in the chick laboratory, where the temperature was 20° to 24°C. during the

[6] A commercial blend of fish oils and vegetable oils containing 1,500 U.S.P. units of vitamin A and 300 units of vitamin D per gram.

January-February series and 24° to 27°C. during the March series.

RESULTS

Table 1 summarizes the results of two typical experiments involving the effect of aureomycin and penicillin on the growth

TABLE 1

Effect of the level of dietary thiamine upon the response of chicks to aureomycin or penicillin [1] (summary of two experiments)

DIET	GROWTH	SURVIVAL [2]	% RESPONSE DUE TO ANTIBIOTICS
	gm		
1.0 μg thiamine·HCl/gm [3]			
Basal	49	12/22	...
Basal + 35 p.p.m. aureomycin	71	19/22	44.9
Basal + 15 p.p.m. penicillin	77	17/22	57.1
1.5 μg thiamine·HCl/gm [4]			
Basal	115	19/22	...
Basal + 35 p.p.m. aureomycin	173	20/22	50.4
Basal + 15 p.p.m. penicillin	195	21/22	69.6
6.0 μg thiamine·HCl/gm [4]			
Basal	211	46/46	...
Basal + 35 p.p.m. aureomycin	231	45/46	9.9
Basal + 15 p.p.m. penicillin	230	45/46	9.0

[1] Dextrin served as the dietary carbohydrate. Chicks were one day old when treatment was begun.

[2] Survival is expressed as the number of chicks surviving the test period divided by the number of chicks started.

[3] Fourteen-day experimental period.

[4] Twenty-one-day experimental period.

of chicks fed varying levels of dietary thiamine. At the adequate level of 6.0 μg of thiamine per gram, the antibiotics increased growth approximately 9.5%. However, at the suboptimum level of 1.5 μg of thiamine per gram, growth was increased 50% by aureomycin and 69% by penicillin. At the lower level of 1.0 μg per gram the antibiotics not only increased growth but survival as well.

The effects of antibiotics on chicks fed or injected with suboptimum amounts of thiamine are shown in table 2. When the

thiamine was fed, aureomycin and penicillin increased growth somewhat, and appeared to decrease the amounts of thiamine and feed required per gram of gain in weight. But when the vitamin was injected, no such responses were observed. Furthermore, growth within the various groups was much more uniform in the injected birds than in those fed thiamine in the diet.

TABLE 2

Effect of aureomycin and penicillin on chick growth when a suboptimum amount of thiamine was supplied in the diet or by injection [1]

DIET	PERFORMANCE				
	At 11 days			At 20 days	
	Chick wt. and std. deviation	gm feed / gm gain	μg B_1 / gm gain	Wt.	μg B_1 / gm gain
	gm			*gm*	
Thiamine·HCl in diet:					
Basal (1.25 μg B_1/gm)	66 ± 22	2.15	2.36	(1)[2]	
Basal + 35 p.p.m. aureomycin	83 ± 16	1.68	1.97	(3)[2]	
Basal + 15 p.p.m. penicillin	78 ± 24	1.70	2.03	(5)[2]	
Thiamine·HCl by injection:[3]					
Basal (no thiamine)	77 ± 9	1.68	1.23	110	1.61
Basal + 35 p.p.m. aureomycin	83 ± 7	1.54	1.05	107	1.71
Basal + 15 p.p.m. penicillin	75 ± 8	1.65	1.22	110	1.64

[1] Dextrin as dietary carbohydrate. Ascorbic acid added at the level of 500 p.p.m. to all diets. Ten chicks per group placed on experiment when one day old.

[2] Number of survivors at 20 days.

[3] Each chick received daily injections of thiamine·HCl totaling 117.5 μg in gradually increasing dosage from 0–20 days, viz., second day, 4 μg per chick; third to 5th days, 5 μg daily; 6th day, 5.5 μg; 7th through 12th days, 6 μg; 13th, 7 μg; 14th to 16th, 8 μg; 17th and 18th, 8.5 μg; 19th day, 9 μg.

In an attempt to diminish variability, 12-day-old chicks were selected for uniform weight gains on a purified diet containing adequate thiamine. The experiment was then repeated with either penicillin or a mixture of penicillin, aureomycin, bacitracin and streptomycin in the diet. When thiamine was fed at the suboptimum level, penicillin increased growth markedly and lessened the dietary requirement for thiamine per gram of gain (table 3). The mixture of antibiotics also in-

creased growth but was no more effective than penicillin alone. As with the newly hatched chicks, these larger chicks showed only a small response to the antibiotics when the level of thiamine in the diet was high, and the antibiotics were also without effect when suboptimum doses of thiamine were given by injection. The original uniformity in the birds was lost

TABLE 3

Effect of penicillin and of a mixture of antibiotics on the growth of older chicks given thiamine in the diet or by injection [1]

DIET	WEIGHT AND STD. DEVIATION AT 30 DAYS	GM FEED / GM GAIN	μG B_1 / GM GAIN
	gm		
Thiamine·HCl in diet:			
Basal (1.5 μg B_1/gm)	236 ± 71	2.60	3.90
Basal + 15 p.p.m. penicillin	310 ± 58	1.97	2.96
Basal + antibiotic mixture [2]	302 ± 62	1.97	2.96
Basal (6.0 μg B_1/gm)	371 ± 34	1.72	10.35
Basal + 15 p.p.m. penicillin	383 ± 35	1.75	10.47
Basal + antibiotic mixture [2]	384 ± 42	1.68	10.10
Thiamine·HCl by injection: [3]			
Basal (no thiamine)	276 ± 18	2.10	1.63
Basal + 15 p.p.m. penicillin	285 ± 18	2.06	1.52
Basal + antibiotic mixture [2]	275 ± 19	2.10	1.63

[1] Dextrin used as dietary carbohydrate. Starting weight 114 gm at 12 days of age; 13 chicks per group. Ascorbic acid added at the level of 500 p.p.m. to all diets.

[2] Antibiotic mixture, in milligrams per kilogram: penicillin 15, aureomycin 25, bacitracin 25, and streptomycin 50.

[3] Thiamine·HCl injected twice daily in gradually increasing dosages, totaling 263 μg per chick for the 12–30 day age period.

when thiamine was in the diet (wide standard deviation) but the responses were quite uniform when the vitamin was given by injection.

Similar results were obtained in subsequent experiments (table 4) in which the carbohydrate was either dextrin or sucrose. In these experiments 1% of glycerol replaced an equivalent portion of the carbohydrate, since thiamine has been shown to be unstable in laboratory diets (Lyman and Elveh-

TABLE 4

Response to penicillin of chicks fed dextrin or sucrose (thiamine fed or injected)[1]

SERIES	AMOUNTS OF THIAMINE GIVEN	DAYS ON EXPERIMENT	AVERAGE WT. AND STD. DEV. OF CHICKS			
			Sucrose		Dextrin	
			No penicillin	Plus penicillin	No penicillin	Plus penicillin
			gm	*gm*	*gm*	*gm*
1	0.6 μg/gm diet	11	67 ± 10	78 ± 18	77 ± 15	89 ± 23
2	0.9 μg/gm diet	16	89 ± 23	100 ± 31	92 ± 35	135 ± 35
1	1.2[2] μg/gm diet	22	232 ± 38	264 ± 36	264 ± 43	287 ± 44
1	6.0 μg/gm diet	22	258 ± 45	255 ± 32	296 ± 39	288 ± 33
2	Subopt., 119 μg total, injected[3]	16	115 ± 14	116 ± 20	122 ± 12	125 ± 12
1	Subopt., 196.5 μg total, injected[4]	22	155 ± 17	150 ± 11	175 ± 14	170 ± 19
1	Opt., 2,440 μg total, injected[4]	22	224 ± 47	234 ± 46	280 ± 21	275 ± 28

[1] One per cent of glycerol added to all diets. Fourteen- and 12-day-old chicks placed on treatment in series 1 and 2, respectively.

[2] Level of thiamine changed to 1.0 μg/gm at 16 days.

[3] No thiamine in diet: thiamine injected twice daily.

[4] No thiamine in diet; thiamine injected once daily.

jem, '51) and glycerol to be protective (Kandutsch and Baumann, '53). Rations were mixed weekly, and stored under refrigeration, and any unused diet from the previous day was discarded.

TABLE 5

Stability of thiamine·HCl in laboratory chick diets as affected by the addition of varying levels of glycerol [1]

% GLYCEROL	% THIAMINE REMAINING		
	Jan.–Feb. series		March series
	Room temp. 53 days	4°C. 53 days	Room temp. 18 days
0	9	55	26
0.25	29	74	
0.50	46	72	
1.0	64	83	57
3.0	76	88	
5.0	90	103	

[1] Sucrose used as dietary carbohydrate. Diets contained 6.0 μg per gram and 2.0 μg per gram of thiamine·HCl in the January–February and March series, respectively.

TABLE 6

Effect of the substitution of glycerol for sucrose on chick growth and feed efficiency [1]

% GLYCEROL ADDED	AVERAGE CHICK WEIGHT AT 4 WEEKS	GM FEED PER GM GAIN
	gm.	
0	342	1.38
1	335	1.32
3	345	1.42
5	330	1.37

[1] Rations of all groups contained an adequate level of thiamine (6.0 μg per gram). Ten chicks per group.

The stabilizing effect of glycerol on the thiamine in purified chick diets is shown in table 5, while table 6 shows that the replacement of the carbohydrate by as much as 5% of glycerol had no adverse effect upon chick growth or feed efficiency. The instability of thiamine in chick diets appears to be at least as serious as that in diets for rats. Since the salt mixture has been implicated with thiamine destruction

(Kandutsch and Baumann, '53), it is noteworthy that 6% of salts are used in chick diets as compared with 4% in diets for rats.

DISCUSSION

The present results do not support the view that antibiotics increase growth by acting directly upon the host (Elam et al., '51), for by this mechanism the response to the antibiotic should have been similar whether the limiting nutrient were fed or injected. A similarity in response would also be expected if the primary function of the antibiotic were to favor the synthesis of the limiting nutrient in the gastrointestinal tract. In the present experiments, however, the antibiotics stimulated growth only when suboptimum amounts of thiamine were present in the diet (average increase in growth of 31.4%); they did not affect the response of chicks to suboptimum amounts of injected thiamine (average increase in growth of 0.68%), and the responses of chicks on a complete diet were also negative or relatively minor (average of 3.75%). These data are in harmony with the concept that the antibiotic suppresses nutrient-utilizing microorganisms in the intestinal tract. The relative variability in the growth of chicks receiving suboptimum amounts of dietary thiamine may be due in part to a variable theft of thiamine by the host's microbial population, and to the fact that ordinary fluctuations in appetite may reduce the intake of thiamine in certain birds, whose food intake then tends to remain low. When thiamine is injected, minor fluctuations in appetite do not affect thiamine intake.

The correction of a minor infection affecting some of the chicks in our experiments was possible to the extent that growth was improved in some cases when antibiotics were added to complete diets. Others (Coates et al., '52; Bird et al., '52; Hill et al., '52) have shown that chicks show a greater response to antibiotics in an environment previously occupied by chickens than in a new environment. "Old" cages

were used in the present study, but the maximum increase in growth due to antibiotics in a complete diet was 9.9% (table 1) and in several series no increases were observed.

The present results may aid in evaluating the relative merits of thiamine given by mouth or by injection. In our earlier experiments (tables 2 and 3) parenteral thiamine appeared to be more efficient than that given orally (less thiamine needed per gram of gain in weight), but this superiority did not persist when special precautions were taken to prevent the destruction of thiamine in the diets. From the growth of the dextrin-fed groups in experiment 2, table 4, it can be calculated that 1.66 and 1.70 μg of injected thiamine were consumed per gram of gain with and without penicillin, respectively, as compared with 1.60 μg of thiamine in the diet containing penicillin and 1.92 μg in the absence of the antibiotic. In other words, dietary thiamine was utilized as efficiently as injected thiamine when losses due to instability *in vitro* or to intestinal microorganisms were avoided.

SUMMARY

1. Penicillin, aureomycin, or a mixture of antibiotics markedly increased the growth of chicks fed diets containing limiting amounts of thiamine. The effect was similar whether the dietary carbohydrate was sucrose or dextrin. When the level of thiamine in the diet was adequate, the antibiotics increased growth only slightly.

2. Dietary antibiotics failed to increase the growth of chicks given suboptimum amounts of thiamine by subcutaneous injection. Variations in weight within groups were much less in the injected birds than in those receiving suboptimum amounts of thiamine in the diet.

3. The presence of 1% of glycerol tended to stabilize the thiamine in semi-purified diets exposed to the conditions of the chick laboratory. Five per cent of glycerol added to the diet had no adverse effect upon growth or feed efficiency.

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THE EFFECT OF LEVEL OF PROTEIN FED BABY PIGS [1]

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Amino acids are among the critical nutrients for the young, growing animal. Maximum utilization of the other components of the ration and maximum rates of gain are dependent upon a continuous supply of amino acids in the proper ratio. The level of protein necessary to provide amino acids in adequate amounts for weanling swine has been a subject of extensive investigation. Little information is available, however, on the level of protein required by pigs of pre-weanling age. Such information would be of value in compounding purified diets for baby pigs, in designing suitable rations for creep feeding and in the preparation of rations most suitable for feeding just after weaning. Purified diets used by investigators of baby pig nutrition have varied considerably in levels of protein and solids (Nesheim and Johnson, '50; Neumann et al., '50; Lehrer et al., '49; Anderson and Hogan, '50; Bustad et al., '48).

The present investigation is concerned with the effect of the level of protein on the growth, nitrogen retention and blood composition of pre-weanling swine.

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EXPERIMENTAL

Rations

The composition of the rations used in these studies, which, insofar as possible, duplicated the composition of swine milk, is given in table 1. The data of Braude et al. ('47), Davis et al. ('51) and Heidebrecht et al. ('51) were used as a basis for compounding the "synthetic milks." Nutrients present in sow's milk in unknown quantity were added at levels found

TABLE 1

Rations used in the level of protein studies[1]

INGREDIENTS	AMOUNT				
Protein (N × 6.25), %	15	20	25	33	41
Vitamin-free casein, gm	15.0	20.0	25.0	35.0	45.0
Lard, gm	47.0	45.5	44.0	41.5	38.0
Lactose, gm	28.0	26.5	25.0	20.5	18.5
Salts,[2] gm	5.0	5.0	5.0	5.0	5.0

[1] The following amounts of vitamins were added to a liter of milk: thiamine 1.1 mg; riboflavin 1.8 mg; nicotinic acid 10.1 mg; inositol 26.8 mg; choline 260 mg; para-aminobenzoic acid 5.0 mg; folic acid 0.13 mg; biotin 0.025 mg; pyridoxine 2.0 mg; calcium pantothenate 7.1 mg; ascorbic acid 130 mg; vitamin A 900 I.U.; vitamin D 120 I.U.; menadione 0.29 mg; alpha-tocopherol acetate 1.5 mg.

[2] Johnson et al. ('48).

acceptable by other workers. The milk was made by dissolving the lactose in hot water, mixing this solution with lard and homogenizing the resulting mixture with a mechanical homogenizer. The protein, salts and vitamins were added to this emulsion with the aid of a Waring Blendor. Changes in the protein content of the ration were made by replacing equicaloric amounts of fat and carbohydrate. The milk contained 20% total solids and was calculated to supply 1,190 calories per liter, using the customary factors to calculate physiological fuel values. The protein in the ration supplied 9.8, 13.1, 16.4, 23.0 and 29.6% of the total calories in the respective rations.

Two experiments were conducted. Six Duroc, two-day-old, litter-mate pigs were used in each experiment. Each experiment consisted of three successive 12-day periods. In the first period a pair was fed one of the three levels of protein. In the second period a pig of each pair was fed one of the remaining two levels of protein, and in the third period each pig received the level of protein it had not received in the previous two periods. In experiment 1, rations containing 15, 20 and 25% protein were fed; in experiment 2, the rations contained 25, 33 and 41% protein. The pigs were fed 4 times a day for the first 4 weeks and three times a day thereafter. All the animals, in each experiment, were fed isocaloric quantities of the ration at each feeding. Daily feed intake records were maintained, and the pigs were weighed every other day. The temperature of the room was maintained at 26°C.

Analytical methods

During the last 6 days of each trial, feces and urine were collected quantitatively and separately every two days, for nitrogen balance determinations. Aliquots of urine and feces were analyzed for nitrogen content by the Kjeldahl method as described by the Association of Official Agricultural Chemists ('45). Nitrogen retention and biological values (Maynard, '47) were calculated, using the data obtained from the nitrogen balance studies. Blood samples were collected every 6 days from the anterior vena cava. Plasma and blood were immediately frozen and stored at — 14°C. for later analyses. Blood samples were assayed for nicotinic acid by the method of Snell and Wright ('41). Tryptophan analyses were made, using the same procedure except that nicotinic acid was added and tryptophan was omitted from the basal media. Hemoglobin was determined by an acid hematin procedure which had been standardized by the method of Wong ('28). Plasma protein was determined by the copper sulfate specific gravity method as described by Hawk et al. ('49).

RESULTS AND DISCUSSION

Table 2 shows the responses of the baby pigs to the various levels of protein in the ration. The weight gains for the 12-day periods were greatest for those pigs fed the highest level of protein in the ration, in each experiment. The nitrogen retained by the pig also increased as the level of protein in the ration was increased. The tendency of the biological value to decrease as the protein content of the ration was increased was particularly evident in the trials in which very young pigs were used. The biological values were calculated in the usual manner and must decrease in magnitude at high nitrogen intakes. Such reduced biological values are not easily correlated with nitrogen retention in rapidly growing pigs. This effect of protein is evident in the ratio of the dry matter consumed to the gain in weight. In general, those animals fed 25% or less protein in the ration required one or more kilograms of dry matter to make 1 kg of gain, whereas those animals fed a level of protein in the ration above 25% required less than 1 kg of dry matter to gain 1 kg.

The regression lines for biological values versus body weight are not colinear between casein levels within experiments 1 or 2. The mean biological values for the 25% level for the two experiments may not be pooled on a statistical basis. The partial regression coefficient of biological value on percentage casein level is — 0.275%. For both experiments, the biological value decreased about 1.11% for each kilogram of body weight increase. This tendency to decrease was statistically significant at the 0.1% level. Bell and Loosli ('51) report that a covariation of biological value and body weight occurs in older pigs.

The efficiency with which the young pigs converted ration dry matter to body weight also decreased with age. This was particularly noticeable in comparing the efficiency of those pigs fed high protein levels, which permitted rapid growth at an early age. For example, in experiment 2 there was an increase in efficiency by pigs receiving the 41% protein ration,

TABLE 2

Response of baby pigs to various levels of protein

	EXPERIMENT 1			EXPERIMENT 2		
	15%	20%	25%	25%	33%	41%
		Period 1			Period 1	
Age, days		12–24			10–22	
Wt. gain, kg	1.38	1.63	1.76	1.29	1.48	1.95
Dry matter eaten/gain, kg	1.16	1.00	0.94	0.99	0.89	0.71
Plasma protein, %	5.8	6.2	6.2	6.5	6.2	6.9
Hemoglobin, %	12.5	13.5	13.2	12.8	12.6	13.6
Plasma tryptophan, μg/ml	10	13	12	11	13	14
Blood niacin, μg/ml	11	10	9	8	8	9
Biological value, %				83	79	76
N retention/day, gm				3.3	4.5	6.1
		Period 2			Period 2	
Age, days		24–36			22–34	
Wt. gain, kg	3.04	3.63	4.28	1.79	2.14	2.43
Dry matter eaten/gain, kg	1.13	0.97	0.84	1.06	0.91	0.84
Plasma protein, %	6.0	5.5	5.9	5.7	6.1	5.7
Hemoglobin, %	12.7	12.3	11.6	11.3	12.2	11.0
Plasma tryptophan, μg/ml	13	15	17	14	15	17
Blood niacin, μg/ml	10	9	9	6	7	7
Biological value, %	91	92	90	82	76	74
N retention/day, gm	9.0	11.9	15.7	6.0	8.0	10.4
		Period 3			Period 3	
Age, days		44–54			34–46	
Wt. gain, kg	4.09	5.56	5.56	3.83	4.20	4.37
Dry matter eaten/gain, kg	1.37	1.03	1.05	0.97	0.91	0.92
Plasma protein, %	5.3	6.1	6.3	5.5	5.4	5.6
Hemoglobin, %	11.7	12.5	12.1	12.5	12.7	13.3
Plasma tryptophan, μg/ml	14	15	15	19	18	17
Blood niacin, μg/ml	8	8	8	7	7	7
Biological value, %	86	85	85	75	71	71
N retention/day, gm	13.5	18.0	22.6	9.2	12.8	16.5

from 0.7 kg dry matter per kilogram gain at two to three weeks to 0.9 kg at 5 weeks.

Results of the hemoglobin, plasma protein, plasma tryptophan and nicotinic acid analyses are summarized in table 3. Different levels of protein in the ration did not appear to influence the hemoglobin, plasma protein or nicotinic acid levels of the blood. There was an increase in plasma tryptophan from 12 μg/ml of plasma at the 15% level to 16 μg/ml at the 41% level. There was also a tendency for the plasma tryptophan level to increase with age. This confirms the previous observation (Reber, '50) that the plasma tryptophan level increased from 10 μg/ml at birth to 15 μg/ml at 4 weeks of age.

TABLE 3

Summary of the responses of the baby pigs to the various levels of protein

	EXPERIMENT 1			EXPERIMENT 2		
	15%	20%	25%	25%	33%	41%
Dry matter eaten/gain, kg	1.22	1.00	0.94	1.01	0.90	0.82
Plasma protein, %	5.7	6.0	6.1	5.9	5.9	6.1
Hemoglobin, %	12.3	12.8	12.3	12.2	12.5	12.6
Plasma tryptophan, μg/ml	12	14	15	15	16	16
Blood niacin, μg/ml	10	9	9	7	7	8

The vitamin-free casein used in these experiments contained 3.3 μg of vitamin B_{12} per gram. Neither vitamin B_{12} nor antibiotics were added to these rations. Anderson and Hogan ('50) reported that vitamin B_{12}, in addition to that contained in alcohol-extracted casein, was necessary to prevent the occurrence of diarrhea under their experimental conditions. No evidence of vitamin B_{12} deficiency was observed in our tests. This may have been due to an adequate level in the ration or to tissue stores.

SUMMARY

The influence exhibited by the level of protein in rations fed baby pigs from two days to 7 or 8 weeks of age was studied, using rate of growth, feed utilization, nitrogen retention

and levels of certain blood components as criteria of response. As the level of protein in the ration was increased from 15 to 41%, the kilograms of dry matter required per kilogram of gain decreased from 1.2 to 0.8; plasma tryptophan increased from 12 to 16 μg per milliliter. Plasma protein, hemoglobin and blood nicotinic acid levels were not significantly altered. Efficiency of conversion of dry matter to body weight showed a negative correlation with age. The biological value of casein decreased as the level of protein in the ration was increased and as the pigs increased in body weight. It appears that the level of protein needed for maximum feed utilization and growth by baby pigs decreases as their body weight increases. The ration containing 41% protein produced maximum weight gains and feed efficiency for the very young pigs. As the pigs approached 8 weeks of age, a level of 20% protein appeared to be used as efficiently as higher levels.

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COMPARISON OF TWO ISOTOPE METHODS FOR DETERMINATION OF ENDOGENOUS FECAL CALCIUM

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TWO FIGURES

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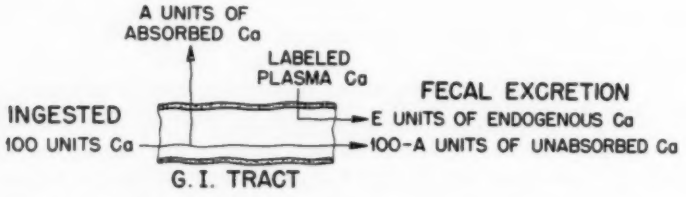
An isotope dilution method for determination of endogenous phosphorus was described and used in 1939 by Hevesy and co-workers (Hevesy, '48). Kleiber et al. ('51) employed the method with dairy cattle and Lofgreen and Kleiber ('53) extended the principles to determination of metabolic fecal nitrogen excretion of young calves by feeding casein labeled with P^{32}. Visek et al. ('53) determined the endogenous fecal calcium in cattle by the same method. Hansard, Comar and Plumlee ('52) and Hansard et al. ('51) presented evidence that in cattle there is a greater retention of ingested radiocalcium than of stable calcium as measured by the usual balance techniques, and suggested the possibility of calculating endogenous calcium from the difference. This did not imply that the stable calcium ions are treated in the body any differently than are the radiocalcium ions. Rather, the data are logically explained by the exchange process which takes place between the calcium ions in blood and bone, and which leads to a selective removal of radiocalcium ions from the blood and deposition in the bone (Comar, Lotz and Boyd, '52). Thus, it becomes possible to estimate endogenous calcium by a method which, although employing the isotope, is independent of the isotope dilution principle. This paper presents for the two methods a comparison of the principles, assumptions, uncertainties, and results with cattle.

EXPERIMENTAL

Principles

Isotope dilution. The basis of the isotope dilution method may be observed in figure 1, which, for convenience, has been based on ingestion of 100 units of dietary calcium. If 100 units of calcium are ingested by an animal and A units are absorbed from the tract, then 100-A will appear in the feces as unabsorbed calcium. If E represents the endogenous calcium for every 100 units of the element ingested, then:

(1) $$\text{Total fecal calcium} = 100 - A + E.$$



Eq. 1. FECAL CALCIUM = 100 - A + E

Eq. 2. $$\frac{\text{SPECIFIC ACTIVITY OF FECAL CALCIUM}}{\text{SPECIFIC ACTIVITY OF PLASMA CALCIUM}} = \frac{E}{100-A+E}$$

Fig. 1 Principle of "isotope dilution" method for calculation of fecal endogenous calcium.

If the plasma calcium, which is the source of the endogenous calcium, is labeled with Ca^{45} and has a specific activity of S_P, then it can be shown that the following relationship holds:

(2) $$\frac{S_F}{S_P} = \frac{E}{100 - A + E},$$

where S_F is the specific activity of the feces. From these two equations the values of the two unknowns, A and E, can be calculated. Expressed narratively, this means that the *labeled* calcium which reaches the feces by the endogenous route is diluted by that which reaches the feces unabsorbed from the diet and which is therefore unlabeled. The extent of this dilution is calculated from the ratio of fecal to plasma specific activities, and the proportion of fecal calcium contributed by each source is thereby determined.

In practice the animal is given a daily injection of Ca^{45} for 10 to 15 days, which builds up a steady state in the secretion of Ca^{45} from the plasma into the feces. The blood and fecal samples for radioassay and chemical analysis are usually taken for several consecutive days during the post-injection period.

Comparative balance. The comparative balance method may be described by reference to figure 2. Using the previous terminology, we may note in figure 2 (I) that if 100 units of calcium are ingested and A units are absorbed, then: (1) total fecal calcium $= 100 - A + E$. It is necessary to consider, however, just what happens to the calcium ions which are absorbed. Unpublished observations in this laboratory and those of other workers (Thomas et al., '52) have shown that every minute about 70% of all the calcium ions in the plasma exchange with those of other compartments. This, together with the fact that about 85% of the Ca^{45} ions put into the blood are shortly found in the bone, means that practically all the calcium ions which are absorbed from the tract will enter the bone and replace similar calcium ions, some of which will be the ones excreted as part of the endogenous calcium. In other words, the endogenous calcium ions have for the most part been involved in bone exchange before being secreted into the gastrointestinal tract.

Let us now consider in figure 2 (II) what happens when 100 units of *labeled* Ca ions are ingested by the same animal. There is no reason to assume that radiocalcium will be absorbed into the blood any differently than will stable calcium, and therefore A units would reach the blood. However, when these radiocalcium ions exchange into bone as do the stable calcium ions, the former will mainly replace stable ions, since the Ca ions in bone contain no Ca^{45} ions at the beginning of the experiment and only a relatively small percentage as the study progresses. This means that the plasma calcium and therefore the endogenous calcium ions will be comprised of relatively few of the radioactive species, a concept which is well-supported by experimental data (Comar et al., '52; Thomas et al., '52). Experiments with intravenously injected Ca^{45} have shown what fraction of the Ca^{45} ions reaching the blood will

appear in the feces, and for present purposes this value has been called B. It follows then that:

$$\text{Fecal } Ca^{45} = 100 - A + BA. \tag{3}$$

The fecal *labeled* calcium will therefore be less than the total fecal calcium. Equations 1 and 3 can be solved for the two unknowns A and E. A convenient form of the equation is:

$$E = \frac{100 - \text{fecal } Ca^{45}}{1 - B} - (100 - \text{fecal calcium}). \tag{4}$$

To obtain the daily endogenous fecal calcium, in grams, it is necessary only to multiply E above by 1/100 of the dietary intake. A sample calculation for steer 8, table 1, is as follows:

$$E = \frac{100 - 72.6}{1 - 0.15} - \left(100 - \frac{13.6}{13.5} \times 100\right) = 33. \tag{5}$$

$$\text{Daily endogenous fecal calcium} = (33)\left(\frac{13.5}{100}\right) = 4.5 \text{ gm}. \tag{6}$$

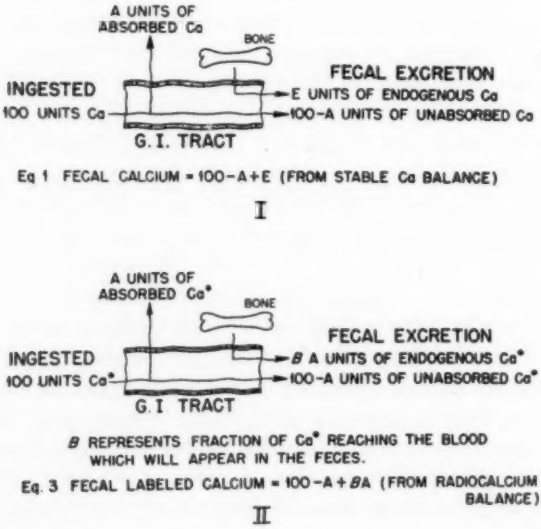

A UNITS OF ABSORBED Ca
BONE
INGESTED
100 UNITS Ca
FECAL EXCRETION
E UNITS OF ENDOGENOUS Ca
100-A UNITS OF UNABSORBED Ca
G.I. TRACT
Eq 1 FECAL CALCIUM = 100-A+E (FROM STABLE Ca BALANCE)
I
A UNITS OF ABSORBED Ca*
BONE
INGESTED
100 UNITS Ca*
FECAL EXCRETION
BA UNITS OF ENDOGENOUS Ca*
100-A UNITS OF UNABSORBED Ca*
G.I TRACT
B REPRESENTS FRACTION OF Ca* REACHING THE BLOOD WHICH WILL APPEAR IN THE FECES.
Eq. 3 FECAL LABELED CALCIUM = 100-A+BA (FROM RADIOCALCIUM BALANCE)
II

Fig. 2 Principle of "comparative balance" method for calculation of fecal endogenous calcium.

Assumptions and uncertainties

Isotope dilution. The fundamental assumption in this method is that the endogenous calcium in the feces, regardless of how it gets there, will have the same specific activity as the labeled calcium of the plasma. This assumption is supported by studies which show that labeled calcium introduced into the bloodstream very shortly comes into equilibrium with the calcium in the soft tissues (Hansard, Comar and Plumlee, '52). Visek (unpublished data) has shown that radiocalcium added to blood *in vitro* comes to equilibrium with the diffusible and non-diffusible fractions of blood calcium within a matter of minutes. It would seem reasonable to assume that equilibrium and steady state conditions would be attained at two to three days after a series of 10 daily injections.

It is important that the fecal and plasma samples are taken at such time that the plasma specific activity represents that of the endogenous calcium in the particular fecal sample. With cattle, it was found by experiment that the fecal specific activity on any one day should be compared with the specific activity of the plasma from the previous day. This time relationship is usually not a great source of error because the change of specific activities with time is very small in the post-injection period.

The limiting errors are probably in the collection and estimation of total fecal calcium.

Comparative balance. The primary assumption in this method is that the labeled calcium, usually given as soluble $Ca^{45}Cl_2$, and all the dietary calcium are absorbed to an equal extent from the intestinal tract. On the basis of the comparative results here reported, there would seem to be little difference between the absorption of calcium in normal plant material and that given as $Ca^{45}Cl_2$. Unpublished data from this laboratory showed no difference in absorption of Ca^{45} from milk as compared with that of $Ca^{45}Cl_2$. Obviously, this method cannot be used if the extent of absorption of the dietary calcium is appreciably different from that of the Ca^{45} administered separately.

The next assumption is that the Ca^{45} which reaches the blood is secreted in the feces to the same extent as that which is intravenously injected. This would be difficult to support experimentally, but it seems reasonable. Also, comparatively large errors in this value of B would not have a large effect upon the final answer. Hansard, Comar and Plumlee ('52) showed that in 12- to 36-month-old cattle the fecal excretion of intravenously administered calcium was remarkably constant at $15 \pm 2\%$. This value, however, does change with age, which must be taken into account in choosing the value for B (Hansard, unpublished data).

The limiting uncertainty is probably in the balance determinations. The variability of the results obtained depends on factors inherent in balance studies — such as the animal's acclimation to the ration and environmental conditions, and the lack of sporadic variations in appetite or bowel movements — as well as on the thoroughness and accuracy of collection and sampling of feces.

RESULTS AND DISCUSSION

The agreement between results obtained by the different methods can best be judged by data from the same animals. As may be noted from table 1, both methods were used with steers 8 and 9. The determinations were run about 20 days apart, with care being taken to see that the animals were managed under similar conditions in each trial. These were Hereford steers on a daily ration of 2.7 lb. corn, 1.3 lb. soybean oil meal, and 8 lb. orchard grass hay. The details for the isotope dilution method have been reported by Visek et al. ('53), and the fecal collections and assays were as described by Hansard et al. ('52). It is clear from the individual values for steers 8 and 9 that excellent agreement was obtained.

Incidental to other studies, endogenous fecal calcium values have been determined in this laboratory on numerous cattle by one or the other of the two methods. In general, the values have been quite consistent. This is shown in table 1, where data are presented from several animals under similar dietary

management and of body weights comparable with those of steers 8 and 9. Cows 1, 6 and 7 were dairy animals and have been described by Visek et al. ('53). The others were Herefords and represent a portion of the animals for which Ca^{45} absorption and tissue distribution have been reported by Hansard et al. ('52). From the over-all averages it would seem

TABLE 1

Endogenous fecal calcium as determined by the "isotope dilution" (I.D.) and "comparative balance" (C.B.) methods

ANIMAL	METHOD	BODY WT.	DAILY Ca INTAKE	DAILY Ca EXCRETED	FECAL EXCRETION OF Ca^{45}	DAILY ENDOGENOUS FECAL Ca
		kg	*gm*	*gm*	%	*gm*
Steer 8	I.D.	397	13	12	...	4.6
Steer 9	I.D.	410	13	15	...	5.2
Cow 1	I.D.	309	55	56	...	5.0
Cow 6	I.D.	288	28	30	...	6.7
Cow 7	I.D.	350	29	20	...	7.2
						Ave. 5.7 ± 0.51 [1]
Steer 8	C.B.	397	13.5	13.6	72.6	4.5
Steer 9	C.B.	410	14.2	15.3	72.1	5.8
Steer 271	C.B.	258	23.7	20.6	73	4.4
Steer 190	C.B.	347	16.2	13.3	59	4.9
Heifer 290	C.B.	314	21.4	22.9	71	8.8
Heifer 151	C.B.	340	42	35	79.3	3.1
Heifer 254	C.B.	396	18.2	13.3	55	4.7
Heifer 264	C.B.	425	17.7	12.2	50	4.9
Heifer 80	C.B.	366	11.6	14.6	77	6.2
						Ave. 5.2 ± 0.53 [1]

[1] The mean ± the standard error.

that both methods gave about the same value for endogenous fecal calcium.

The primary advantage of the isotope dilution procedure is that it is independent of the availability of the dietary calcium. In fact, it may be used to give an estimate of the true availability of various sources of calcium. An advantage of the comparative balance method is that it may be useful in cases where sufficient blood samples are not obtainable.

The number of analyses required for each method is about the same. The isotope dilution method requires about 10 daily injections of radioactive material and then calcium and Ca^{45} analyses on about 5 to 7 plasma and 5 to 7 feces samples. If the only information required is the proportion of fecal calcium that is endogenous, then no records need be kept of the total calcium input or outgo. If the actual endogenous value in grams is required, then it is necessary to measure also the total calcium excretion. If it is desired to calculate the "true digestibility," then the total calcium intake must also be known. The comparative balance method requires measurements of calcium intake, total fecal calcium, and the total excretion of an orally administered dose of Ca^{45}. It is also necessary to know, for the particular animal, the percentage of intravenously injected Ca^{45} which will reach the feces. One source of uncertainty may be the fact that the labeled calcium excretion occurs mainly over a given two- or three-day period, whereas it is felt that at least a 7-day balance period is required for a meaningful stable calcium balance.

The basis of the comparative balance method should emphasize the careful attention that must be given to interpretations of the behavior of a stable substance in a system, as deduced from experimental observations of the tagged substance.

SUMMARY

Comparable values have been obtained for endogenous fecal calcium in cattle by an "isotope dilution" method and a "comparative balance" method. Both procedures involve the use of labeled calcium, but they are different in principle. The "isotope dilution" method offers advantages in being independent of the availability of the dietary calcium and of the uncertainties of balance determinations.

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DELAYED PROTEIN SUPPLEMENTATION OF CORN DIETS FOR GROWING SWINE [1]

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INTRODUCTION

During recent years much interest has been shown in the time element with respect to protein supplementation. Berg and Rose ('29) demonstrated that when rats fed a tryptophan-deficient diet were given supplemental tryptophan at varying intervals, the growth rate decreased as the intervals between feedings of the amino acid (tryptophan) increased to more than 12 hours. It has since been demonstrated that when the feeding of missing tryptophan, lysine or methionine is delayed until several hours after an "incomplete" mixture of amino acids is consumed, growth is not supported (Geiger, '47). Henderson and Harris ('49) showed that lysine supplementation delayed three to 7 hours after feeding of a lysine-deficient diet gave significantly lower nitrogen retention, body weight gain, weight gain per gram of diet eaten, and weight gain per gram of lysine eaten than when not delayed. These differences were greater as the interval between supplementary lysine feedings lengthened. It has also been shown that pairs of complementary whole proteins fed to rats al-

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ternately on opposite days gave lower biological values and growth than when they were fed together (Henry and Kon, '46), and when they were fed alternately for 10-hour periods, with two-hour lapses before alternating the diets, growth was not promoted (Geiger, '48a, b). Leverton et al. ('51) demonstrated with human subjects that omission of animal protein from the noon meal adversely affects nitrogen utilization when the daily protein intake is as low as 43 gm, but does not affect it when the intake is 63 gm. No reports of similar work with swine were found.

EXPERIMENTAL

Weanling barrow pigs were used in all experiments. They were weaned at approximately 8 weeks of age and were kept on a standard ration for a week or more prior to being placed on experiment. They were allotted as evenly as possible into groups of 4 pigs each, according to size and genetic background. All pigs were weighed weekly and individual feed intakes were adjusted to equal 4.5 or 5.0% of body weight per day. They were all individually fed twice daily. Nitrogen balance data were obtained, using 7- or 8-day preliminary feeding periods followed by 5- or 6-day collection periods.

Experiments 1 and 2 were of a single reversible design; two methods of feeding were compared in each case. In each experiment the protein supplement and corn were fed to give a diet containing approximately 17% of protein. The normal feeding method in all cases consisted of feeding the corn and supplement as a mixed ration. The other feeding method in experiment 1 consisted of feeding all of the day's protein supplement plus some corn at the morning feeding, and corn alone at the evening feeding. In experiment 2 the interval between feedings of supplemental protein was increased to 36 hours, with two feedings of corn fed alone intervening between feedings of the supplement. Equal amounts of total feed were fed at morning and evening feedings. The pigs usually would consume all of a feeding in about 30 minutes. Table 1 shows

TABLE 1

Composition of protein supplement mixture

(Experiments 1 and 2)

CONSTITUENTS	AMOUNT
	%
Soybean oil meal (41% protein)	75.1
Alfalfa meal (dehydrated)	15.0
Vitamins [1]	2.8
Mineralized salt [2]	1.4
Steamed bonemeal	3.4
Ground limestone	1.7
Merck B_{12} supplement 622 [3]	0.3
Bi-Con TM5 [4]	0.3

[1] Furnished riboflavin, 550 mg; pantothenic acid, 2.2 gm; niacin, 3.3 gm; choline, 150 mg per kilogram of vitamin mixture.

[2] Furnished additions of iodine, iron, copper, cobalt, manganese and zinc.

[3] Furnished 27.5 mg of vitamin B_{12} per kilogram.

[4] Commercial supplement furnished by Chas. Pfizer and Co., furnishing 11 gm of terramycin per kilogram.

TABLE 2

Composition of protein supplement mixture

(Experiment 3)

CONSTITUENTS	AMOUNT
	%
Soybean oil meal (51% protein)	32.7
Tankage	18.7
Fish meal	14.0
Alfalfa meal (dehydrated)	18.7
Blood flour	9.4
Ground limestone	2.3
Steamed bonemeal	0.7
Salt	2.3
Aurofac [1]	1.2
Vitamins [2]	+

[1] Commercial supplement furnished by Lederle Laboratories furnishing 4.0 gm of aureomycin and 4.0 mg of vitamin B_{12} per kilogram.

[2] Furnished riboflavin, 11.0 mg; niacin, 55.0 mg; and calcium pantothenate, 55.0 mg per kilogram of supplement. Sufficient amounts of vitamin D were given weekly to furnish approximately 110 I.U. per kilogram of diet consumed.

the composition of the protein supplement used in the first two experiments.

In experiment 3, 4 feeding methods were compared: normal feeding and 24-hour, 36-hour and 48-hour intervals between feedings of the supplement. Two replicates of 4 pigs each were used, each replicate forming a separate 4×4 Latin square. Table 2 shows the composition of the protein supplement used in experiment 3. The protein supplement was fed with corn to give a diet containing approximately 16% of protein. Both protein supplements used in the three experiments were designed to correct the lysine and tryptophan deficiencies of corn protein.

RESULTS AND DISCUSSION

The average performance data from experiments 1 and 2 are presented in table 3. The percentage of apparently digested nitrogen retained from the normal feeding was 45.0% as compared with 44.0% retention when the 24-hour interval intervened between supplemental protein feedings. Daily gains and feed efficiency were also similar for the two methods of feeding. With daily energy and protein intakes the same, the 24-hour interval between supplemental protein feedings did not decrease the utilization of the total protein. In experiment 2, the average percentage of apparently digested nitrogen retained from the normal feeding was 47.8%, as compared with 44.1% retention for the 36-hour interval between supplemental protein feedings. This difference was not statistically significant. Corresponding daily gains and feed efficiencies were similar for the two methods of feeding.

The average performance data from experiment 3 are presented in table 4. The values for apparently digested nitrogen retained from the normal feeding and from feedings with a 24-hour interval between protein supplements were 51.9 and 51.8%, respectively. The values for the 36-hour and 48-hour intervals between feedings of the protein supplement were 48.3 and 44.8%, 7 and 14% lower, respectively, than the nitrogen retention obtained with the normal feeding.

An analysis of variance of the data (replicates 1 and 2 combined) showed that the differences in nitrogen retention due to the feeding methods were significant ($P \leq 0.05$). Bell and Loosli ('51) have demonstrated that biological values tend to decrease as pigs increase in size. Covariance analysis removed some variation in the nitrogen retention values associated with increases in the size of the pigs in this experiment, but the difference due to feeding methods did not reach the 1% level of significance.

TABLE 3

Experiments 1 and 2 — Average performance

CATEGORY OF INTEREST	EXPERIMENT 1		EXPERIMENT 2	
	Normal feeding	24-Hour interval	Normal feeding	36-Hour interval
Number of pigs	4	4	4	4
Initial weight (kg)	19.6	20.0	31.7	31.5
Daily feed intake (% of body weight)	4.70	4.70	4.28	4.19
Daily gain (gm)	378	371	640	640
Daily gain (% of initial wt.)	1.91	1.82	2.01	2.02
Grams gained per kg of feed	358	342	406	415
Apparent digestibility of nitrogen (%)	74.9	74.2	80.0	79.0
Apparently digested nitrogen retained (%)	45.0	44.0	47.8	44.1

As measured by least significant difference, the 14% decrease in nitrogen retention obtained for the 48-hour interval between feedings of supplemental protein was significantly ($P \leq 0.05$) lower than that obtained for the normal feeding or for the 24-hour interval between supplemental protein feedings. The 7% reduction in nitrogen retention obtained for the 36-hour interval between supplemental protein feedings was not significantly lower than that for the normal feeding.

The differences in daily gains due to time of protein supplementation were highly significant ($P \leq 0.01$), according to an analysis of variance of the gains expressed as a percentage of the initial weight. For the analysis the corresponding angles for these values as given by arc sign tables (Snedecor, '46) were used. The daily gains for the 36-hour and 48-hour intervals between feedings of supplemental protein were significantly lower ($P \leq 0.01$) than that for the normal feeding

TABLE 4

Experiment 3 — Average performance
(Replicates 1 and 2 combined)

CATEGORY OF INTEREST	NORMAL FEEDING	INTERVAL BETWEEN FEEDINGS OF PROTEIN SUPPLEMENT		
		24 hours	36 hours	48 hours
Number of pigs	8	8	8	8
Initial weight (kg)	28.4	28.9	30.0	30.0
Daily feed intake (% of body weight)	4.15	4.13	4.14	4.16
Daily gain (gm)	535	522	505	489
Daily gain (% of initial wt.)	1.88	1.81	1.64	1.60
Grams gained per kg of feed	393	383	350	342
Apparent digestibility of nitrogen (%)	80.1	79.6	80.1	79.5
Apparently digested nitrogen retained (%)	51.9	51.8	48.3	44.8

by least significant difference analysis. These daily gains were also significantly lower ($P \leq 0.05$) than that for the method of feeding in which the protein supplement was fed at 24-hour intervals, approaching the 1% level of significance very closely.

From the results of these experiments it appears that weanling pigs utilize the proteins from corn and a mixed supplement nearly as efficiently if the supplement is fed at every other feeding (24-hour intervals) as if it is fed at each feed-

ing (twice daily). It is assumed that the proteins of the corn were being effectively supplemented by the proteins in the supplement used. Other work at this station (Eggert et al., '53) indicates that this is probably true. Under similar experimental conditions, the retention of apparently digested nitrogen from corn protein alone varied from 30 to 35%. The lack of difference between the results obtained with normal feeding and those resulting with the 24-hour interval between feedings of supplemental protein is not consistent with results reported in the literature for similar experiments with rats. It is pointed out that the proteins of the supplemental mixture and the corn used in this experiment were more varied and complex than those used in most of the rat experiments. A slower, more gradual release of amino acids from these proteins may have extended over a long enough period to allow mutual metabolic supplementation regardless of the 24-hour interval between feedings of the protein supplement.

If the above is a valid explanation for the lack of difference in nitrogen utilization between the normal feeding method and the method in which the interval between feedings of protein supplement was 24-hours, it is still surprising that the 48-hour interval allowed as effective mutual supplementation as was evidenced. Possibly protein metabolism and its relationship to energy metabolism in the pig are different enough from those in the rat to explain the unexpected results. The ingested feed generally passed through the digestive tract within 20 to 30 hours, as indicated by the carmine marker.

It is evident, however, that increasing the interval between feedings of supplemental protein to as long as 48 hours did considerably decrease the net utilization of the mixture of proteins. It is not felt that all of the greater nitrogen retention obtained with normal feeding can be attributed to the nitrogen-sparing effect of carbohydrate when fed with protein (Monro, '49; Geiger, Bancroft and Hagerty, '50), inasmuch as the protein supplements which were fed contained considerable carbohydrate and at least 20% of corn was included in all of the protein supplement feedings.

SUMMARY

Experiments were conducted to test the effect of the time of protein supplementation of corn diets on total protein utilization of male weanling pigs, as measured by growth rate and nitrogen balance.

Growth was as rapid and nitrogen utilization was as efficient for feedings with a 24-hour interval between supplements of protein as for the normal method of feeding. The 36-hour and 48-hour intervals between feedings of the protein supplement caused 7 and 14% decreases in nitrogen retention, respectively. Although the 48-hour interval between feedings of supplemental protein caused lower total protein utilization, it is estimated that 40 to 50% more efficient utilization was obtained than had corn alone been fed under similar conditions, indicating some mutual supplementation of the corn and supplemental proteins.

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BLOOD AND BONE MARROW STUDY OF VITAMIN-DEFICIENT RATS [1]

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Shukers and Day ('43) have reported that riboflavin deficiency and inanition not only caused leukopenia but also led to an absolute and a relative decrease of lymphocytes in the peripheral blood of rats. Berry et al. ('45) demonstrated that a total vitamin B complex deficiency resulted in a relative increase in neutrophiles and a relative decrease in lymphocytes in the peripheral blood of rats which were fed a non-synthetic basal diet.

These changes may be due to the hematopoietic response of the bone marrow which serves as a source from which the peripheral blood supply is replenished. There is general agreement that erythrocytes, granulocytes and thrombocytes have their origin in the hematopoietic tissue of the bone marrow. Therefore, the purpose of this investigation was to determine whether or not hematopoietic changes in the peripheral blood are accompanied by corresponding changes in the bone marrow of totally vitamin B complex-deficient rats, thiamine-deficient animals and inanition control rats. A purified synthetic basal diet was employed.

EXPERIMENTAL

Male, weanling, albino rats of the Sprague-Dawley strain were distributed into groups as indicated in the tables. The

[1] This investigation was supported by a research grant from the Upjohn Laboratories, Kalamazoo, Michigan.

rats were housed individually in wide-meshed, screen-bottom cages to prevent coprophagy. All the animals were weighed daily and the weights recorded.

The ingredients [2] used for the preparation of the basal diet and the vitamin pills were of the highest purity obtainable. The basal diet consisted of the following, in per cent: sucrose, 65.12; vitamin-free casein, 17.23; hydrogenated vegetable oil, 9.55; salt mixture no. two, 3.88; cod liver oil,[3] 2.00; corn oil,[4] 2.00; choline chloride, 0.20; *i*-inositol, 0.01; para-aminobenzoic acid, 0.01; and 2-methyl-1, 4-naphthoquinone, 0.01.

The thiamine-deficient and totally vitamin B complex-deficient animals were fed the basal diet ad libitum, and each inanition control animal was fed the amount of basal diet sufficient to maintain its weight at a level equal to that of its deficient partner. With the exception of the totally vitamin B complex-deficient group, each rat was fed a vitamin pill daily. The control animals received a pill containing the following vitamins (Griffith and Ferris, '49): thiamine, 40 μg; riboflavin, 60 μg; nicotinic acid, 150 μg; calcium pantothenate, 150 μg; pyridoxine, 50 μg; folic acid, 1 μg; and biotin, 1 μg. Thiamine was omitted from the pills fed to the thiamine-deficient rats.

Manifestation of typical deficiency symptoms became apparent after a 4-week feeding period. The animals on the thiamine-deficient diet did not gain any appreciable weight and showed symptoms of paraplegia and convulsions. Those on the vitamin B complex-deficient diet displayed similar symptoms, plus a loss of hair. At this time the tip of each rat's tail was cut to obtain blood for the total erythrocyte, total leukocyte and differential cell counts. Wright's staining method was employed for the differential cell count preparations (Kolmer and Boerner, '45). Two hundred cells were

[2] Obtained from General Biochemicals, Inc., and Nutritional Biochemicals Company.

[3] Squibb.

[4] Mazola.

observed and counted for each preparation. Duplicate counts were made from the blood smear of each animal.

The rats were anaesthetized and from each one the proximal portion of the tibia was removed. Bone marrow films were prepared by diluting the marrow with normal rabbit serum according to the method of Endicott ('45). Wright's staining technic was employed and 500 cells from each slide were counted and classified.

RESULTS AND DISCUSSION

At the end of the 4-week feeding period, the ad libitum control rats had gained weight, appeared healthy and possessed smooth, even coats. The inanition control rats, although their food intake had been restricted, appeared healthy and possessed smooth, even coats. The thiamine-deficient and totally vitamin B complex-deficient animals displayed symptoms typical of their respective deficiency. The initial average and final average weights of each group are recorded in table 1.

Individual blood counts were performed and the results were recorded as the range and median for each group (table 1). The results indicate that the vitamin deficiencies and inanition resulted in a marked leukopenia. No significant effect was observed on the total erythrocyte count in the peripheral blood of the groups studied.

A change in the differential cell count of peripheral blood was noted in the thiamine-deficient, totally vitamin B complex-deficient and inanition groups. Even though there was a severe reduction of total leukocytes per cubic millimeter in the deficient rats, the number of polymorphonuclear neutrophiles per cubic millimeter was not appreciably altered. This is accounted for by a percentage increase of these cells in the deficient animals. However, a marked reduction in the percentage and number of lymphocytes per cubic millimeter was observed. The median values for granulocytes in the deficient animals and inanition controls were not significantly different from those in the ad libitum controls. However, individual animals had polymorphonuclear neutrophile counts as low as

TABLE 1

Peripheral blood counts of thiamine-deficient, vitamin B complex-deficient, inanition and ad libitum control rats

DIET AND NO. OF RATS		RBC 1×10^6	WBC	DIFFERENTIAL CELL COUNT				AVE. BODY WT.	
				Lymph.		Poly.		Init. ave.	Final ave.
		cells/mm³	*cells/mm³*	%	*cells/mm³*	%	*cells/mm³*	*gm*	*gm*
Thiamine-deficient group (10)	Range	9.36–11.37	2,850–11,700	28.5–71.5	1,280–7,840	28.5–71.5	810–4,000	37.6	43.7
	Median	10.02	4,900	57.0	2,450	38.5	3,220		
Totally vitamin B-deficient group (12)	Range	6.94–12.80	3,650–8,450	43.0–76.5	1,970–4,220	23.0–56.0	920–4,730	38.4	38.5
	Median	9.75	5,750	51.5	3,350	42.5	2,560		
Inanition control group (14)	Range	8.33–14.20	3,250–9,900	49.0–90.0	1,600–6,930	9.0–50.5	380–2,910	37.4	42.6
	Median	10.82	4,250	66.5	3,780	31.5	1,560		
Ad libitum control group (6)	Range	8.56–10.16	16,800–23,150	70.0–92.0	13,900–21,300	8.0–28.5	1,500–5,700	36.2	115.0
	Median	9.23	18,400	85.0	14,200	11.5	2,200		

380 per cubic millimeter. The lowest value in the ad libitum controls was 1,500 per cubic millimeter. The monocytes, eosinophiles and basophiles were found to be normal and are not reported in table 1.

The cellular composition of the blood is intimately associated with the composition of the bone marrow. Under normal physiologic conditions, the relative numbers of the dif-

TABLE 2

Cellular composition of the bone marrow in vitamin-deficient rats, inanition control animals, and normal rats

BONE MARROW CELL		DIET AND RELATIVE CELLULAR PERCENTAGE			
		Thiamine-deficient	Totally vitamin B-deficient	Inanition control	Ad libitum control
		%	%	%	%
Total granulocytes	Range	34.6–67.8	27.8–72.2	24.8–54.2	26.6–48.0
	Median	56.4	49.2	41.1	32.7
Promyelocytes and myelocytes	Range	7.6–17.4	4.2–21.4	4.8–15.2	3.6–14.0
	Median	15.2	10.0	12.0	9.1
Metamyelocytes and segmenters	Range	26.0–46.8	23.0–43.0	19.6–33.2	20.8–27.6
	Median	39.0	37.2	26.6	20.1
Eosinophiles	Range	1.0–3.6	0.6–7.8	0.4–5.8	2.2–6.4
	Median	2.2	2.0	2.5	3.5
Nucleated red blood cells	Range	28.6–54.0	27.8–52.6	40.0–61.6	44.0–64.6
	Median	33.8	44.0	51.8	47.8
Lymphocytes	Range	1.8–4.6	1.2–5.0	1.2–5.0	1.6–12.0
	Median	2.4	3.0	3.0	11.1
Blast cells	Range	0.8–3.8	0.4–2.6	0.4–2.0	0.4–3.2
	Median	1.4	1.2	1.0	1.3
Mast cells	Range	0.4–3.4	0.4–2.6	0.0–2.0	0.2–1.4
	Median	1.8	0.8	1.0	0.5
Plasma cells	Range	0.6–2.2	0.4–2.8	0.4–2.0	0.4–1.4
	Median	1.8	1.0	0.9	0.8
Monocytes	Range	0.2–1.2	0.2–1.2	0.2–1.6	0.2–1.0
	Median	0.6	0.8	0.8	0.6
Unclassified	Range	1.4–3.8	0.4–6.0	0.6–3.0	0.2–1.8
	Median	2.2	1.2	1.3	1.1

ferent types of cells in the bone marrow, as well as in the peripheral blood, vary only to a minor degree. However, due to certain abnormal physiologic conditions, changes in the bone marrow are usually accompanied by changes in the cellular composition of the blood in the peripheral circulation.

The bone marrow of rats with thiamine deficiency and total vitamin B complex deficiency showed a marked relative granulocytosis with a corresponding increase in the younger myeloid cells when compared with the marrow of the ad libitum control animals. The bone marrow of the vitamin-deficient and the inanition control rats demonstrated a relative lymphocytopenia and a slight increase in granulocytes. The changes in the number of nucleated red cells in the bone marrow were almost as great as those which occurred with respect to the total granulocytes. A lower median value for nucleated red cells in the thiamine-deficient group was obtained. The percentages of the other types of cells classified were not significantly different from those of the ad libitum controls. The average relative per cent of each type of cell appears in table 2.

SUMMARY

1. This study indicates that leukopenia occurred in the peripheral blood of the thiamine-deficient, totally vitamin B complex-deficient and inanition control rats.

2. A percentage increase in the polymorphonuclear neutrophiles, and a percentage and a total number decrease of lymphocytes in the peripheral blood of these animals, were observed. The number of neutrophiles per cubic millimeter was not appreciably changed.

3. An increased percentage of granulocytes was observed in the bone marrow of thiamine-deficient and totally vitamin B complex-deficient animals. The bone marrow from the inanition control animals likewise demonstrated granulocytosis, but to a lesser degree than that of the vitamin-deficient animals.

4. A lower median value for nucleated red cells in the thiamine-deficient group was obtained from the study of the bone marrow smears.

5. A marked relative lymphocytopenia was observed in the bone marrow of thiamine-deficient, totally vitamin B complex-deficient and inanition control animals.

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THE EFFECTS OF PROCESSING TEMPERATURES ON THE AMINO ACID CONTENT OF SUNFLOWER SEED OIL MEAL[1]

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In a recent communication, Morrison, Clandinin and Robblee ('53) reported that high processing temperatures adversely affect the nutritive value of sunflower seed oil meal for chicks. Using the microbiological technique, Riesen, Clandinin, Elvehjem and Cravens ('47) demonstrated that subsequent to acid hydrolysis (alkaline hydrolysis in the case of tryptophan) less lysine, arginine and tryptophan were liberated from over-heated soybean oil meal than from properly-heated soybean oil meal. In the work reported herein, a study was made on the effects of processing temperatures on the amino acid content of sunflower seed oil meal.

EXPERIMENTAL

Three sunflower seed oil meals (designated S1, S2 and S3) were obtained from a commercial processing plant.[4] Meals S1 (processed at 200°F. in the cooker and 220°F. in the conditioner) and S2 (processed at 220°F. and 240°F.) were shown (Morrison et al., '53) to be of superior nutritive value to meal S3 (processed at the regular processing temperatures

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of 240°F. in the cooker and 260°F. in the conditioner). A 4th meal (S4), prepared by heating meal S1 in the autoclave for 4 hours at 15 pounds' steam pressure, was reported to be of decidedly inferior nutritive value for chicks.

Using the microbiological technique, a study was made on the liberation of essential amino acids (glycine excluded) from the meals by acid (alkaline hydrolysis in the case of tryptophan) and enzymatic hydrolysis. The hydrolysis procedures, test organisms and basal media employed were the same as those used by Clandinin ('49). Assays were conducted on a semimicro scale (2 ml per tube). A Cannon Automatic Dispenser-Titrator was used in connection with the assay work.

RESULTS AND DISCUSSION

The results (table 1), expressed as percentage amino acid liberated from protein calculated by nitrogen × 6.25, were calculated from the averages of three assays in duplicate.

TABLE 1

Liberation of essential amino acids [1] from sunflower seed oil meals

HYDROLYSIS Meal number [3]	ACID [2]				ENZYMATIC			
	S1	S2	S3	S4	S1	S2	S3	S4
Lysine	3.27	3.18	2.78	1.98	1.57	1.92	1.71	0.80
Arginine	9.37	9.29	8.70	6.83	6.83	9.20	9.80	6.92
Tryptophan	1.04	1.01	0.97	0.81	0.53	0.91	1.02	0.84
Methionine	1.55	1.65	1.53	1.61	1.12	1.37	1.50	1.20
Histidine	2.11	2.10	2.09	1.91	1.03	1.46	1.60	1.29
Threonine	3.20	3.28	3.16	3.12	1.78	2.49	2.65	2.02
Leucine	6.14	5.93	5.88	5.94	2.82	4.52	5.08	4.34
Isoleucine	3.97	3.98	3.90	3.90	1.87	2.87	3.22	2.84
Valine	4.85	4.99	4.89	4.82	2.00	3.32	3.74	3.25
Phenylalanine	4.20	4.23	4.26	4.16	1.75	3.17	3.70	3.33
Total	39.70	39.64	38.16	35.08	21.30	31.23	34.02	26.83

[1] Expressed as percentage amino acid liberated from protein calculated by nitrogen × 6.25.

[2] Alkaline hydrolysis for tryptophan.

[3] See section headed "Experimental" for description of meals.

Statistical analysis of acid hydrolysis values yielded minimum significant differences as follows: lysine 0.10, arginine 0.44, tryptophan 0.05.

With the exceptions of lysine, arginine and tryptophan, the data show little difference in the liberation of essential amino acids subsequent to acid hydrolysis (alkaline hydrolysis for tryptophan) of meals S1, S2 and S3. Indirectly, the data indicate that rations containing meals S1 or S2 as the only source of supplementary protein would be marginal in lysine content for the chick. Hence, the slower rate of growth of chicks fed meal S3 as compared to that of chicks fed meals S1 and S2 observed by Morrison et al. ('53) may have been the result of the slight destruction of lysine (15%) during the processing of meal S3.

On the basis of the very poor growth results obtained from meal S4 (Morrison et al., '53), one might expect an appreciable decrease in the liberation of essential amino acids by acid hydrolysis of meal S4 (alkaline hydrolysis for tryptophan), as compared to the liberation from meal S1. Depressed liberation of lysine (40%), arginine (27%) and tryptophan (21%) was noted. These results, in the main, parallel those obtained by Riesen et al. ('47) on over-heated soybean oil meal.

There appears to be no relationship between the liberation of essential amino acids by enzymatic hydrolysis of meals S1, S2, S3 and S4, and their nutritive value. The results show greater liberation of essential amino acids from meal S3 than from meal S1, although meal S1, as previously stated, is of superior nutritive value to meal S3. In addition, although sunflower seed oil meal S4 is of decidedly inferior nutritive value for chicks, the liberation of essential amino acids (lysine excluded) by enzymatic hydrolysis of meal S4 was as great or greater than the liberation from meal S1.

SUMMARY

A study was made, using the microbiological technique, on the liberation by acid (alkaline hydrolysis for tryptophan) and enzymatic hydrolysis of essential amino acids from 4 sunflower seed oil meals processed under different conditions. The results obtained indicated that:

1. Less lysine, arginine and tryptophan were liberated by acid hydrolysis from a regular-temperature sunflower seed oil meal (processed at 240°F. in the cooker and 260°F. in the conditioner) than from low- or medium-temperature sunflower seed oil meals (processed at 200°–220°F. and 220°–240°F., respectively).

2. Heating low-temperature sunflower seed oil meal in an autoclave for 4 hours at 15 pounds' steam pressure resulted in depressed liberation by acid hydrolysis (alkaline hydrolysis in the case of tryptophan) of lysine, arginine and tryptophan.

3. No relationship exists between the liberation of essential amino acids by enzymatic hydrolysis and previously reported relative nutritive values for the sunflower seed oil meals.

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